GENETIC AND ENVIRONMENTAL DETERMINANTS OF RESPIRATORY HEALTH

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Genetic and environmental determinants of respiratory health

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CHAPTER 1

General Introduction

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GENERAL INTRODUCTION

Respiratory symptoms

Cough, dyspnea, phlegm, and wheeze are common respiratory symptoms, which have been regarded as important markers of the risk of having or developing respiratory disease and are associated with accelerated lung function decline (1-3), impaired quality of life (4, 5), hospitalization (4, 5), asthma (6, 7), COPD (3, 8), and even mortality (9-12). The prevalence of respiratory symptoms ranged from 3% to 32% among children and from 1% to 35% among adults in the European Community Respiratory Health Study (ECRHS) and the prevalence of respiratory symptoms is still increasing (13, 14). Although respiratory symptoms can occur in individuals of all ages, the risk of suffering from respiratory symptoms increases with age (15). In children, the prevalence of respiratory symptoms (16-19) and asthma (20) is higher in boys than in girls, while in adults females have a higher prevalence (16-19). In addition, respiratory symptoms are associated with lower lung function independent of respiratory diseases (21).

Common respiratory symptoms

The most common respiratory symptoms are cough, dyspnea, phlegm and wheeze.

Cough

Cough is a sudden and often repetitively occurring reflex action of the respiratory tract that is used to clear secretions such as mucus, irritants, foreign particles and microbes in the upper airways (22). A respiratory tract infection is the most common cause of acute cough. Chronic cough, can be triggered by smoking, air pollution and occupational exposures, and is a common symptom of several chronic respiratory conditions such as asthma, chronic obstructive pulmonary disease (COPD), and lung cancer (23), but is also present in non-respiratory conditions such as heart failure (24).

Dyspnea

According to the American Thoracic Society definition, dyspnea is a subjective experience of breathing discomfort that consists of qualitatively distinct sensations that vary in intensity (25). Other definitions describe dyspnea as “difficulty in breathing”, “uncomfortable awareness of breathing” and “shortness of breath” (26). Healthy subjects can experience dyspnea in specific situations, e.g. at high altitude, after breath holding, during stressful situations and during strenuous exercise (27). Dyspnea is considered pathologic and a symptom of a disease if it occurs at rest or walking at own pace (28). It is worth to mention that dyspnea is a common symptom not only in patients with lung and heart diseases, but it is also fairly prevalent among elderly individuals without apparent pre-existing disease (10, 28). Obesity is an important risk factor for dyspnea (29).

Phlegm

Phlegm is a secretion produced by the mucous membranes of the respiratory tract (throat or larynx) during disease and inflammation. It mainly consists of mucus with bacteria, debris, small inhaled particles, inflammatory cells and necrotic epithelial cells (30). Phlegm becomes more excessive during bacterial or viral infections such as acute bronchitis, influenza, cold, and pneumonia (30, 31). Smoking is the most important environmental risk factor for phlegm (32).

Wheeze

Wheeze is a continuous and high-pitched whistling sound produced in the respiratory airways during breathing (33). It is caused by narrowed airways and/or inflammation. The most common cause of recurrent wheezing is asthma. However, there are many other potential causes for wheezing which may also be an indication of COPD, emphysema, sleep apnea, heart failure and lung cancer (34). Both smoking and BMI can contribute to wheeze (32).

Lung function

The level of lung function is age-, height- and sex dependent. Lung function increases during lung development and growth in neonate and during childhood. The peak period is approximately between 20 and 30 years of age. After the age of 30, lung function starts to decline as part of the normal aging process. Abnormally accelerated decline of lung function is seen in respiratory diseases and may occur at several stages of life (35, 36).

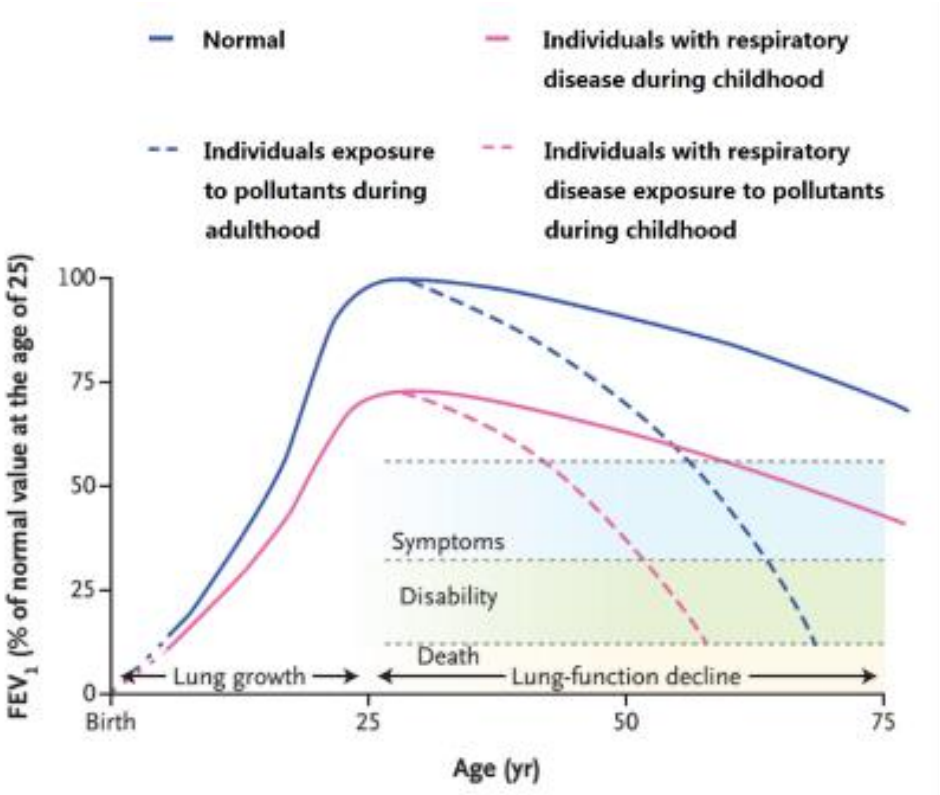

Figure 1. Level of lung function and symptoms during the life-span (adapted from Brusselle, 2009) (37).

Environmental risk factors for respiratory symptoms and level of lung function

Respiratory symptoms are common and complex physiological phenomena with various possible causes. The most important environmental risk factors of respiratory symptoms are cigarette smoking (38, 39), air pollution (40-42), and occupational exposures (43-45). Environmental tobacco smoke exposure induces inflammation and

oxidative stress in the lungs and has been associated with reduced levels of lung function at birth and in adulthood, as well as respiratory symptoms (46, 47). Apart from smoking, exposure to air pollution (a mixture of hundreds of pollutants originating from industry, traffic, heating and other sources) has been shown to lead to increased respiratory symptoms. Air pollution is therefore considered to be another environmental risk factor for respiratory symptoms (40, 48). Occupational exposures such as organic and inorganic dusts, chemical agents and fumes are associated with respiratory symptoms (49, 50). However, not all individuals with exposure to smoke, air pollution or occupational exposures develop respiratory symptoms, suggesting that there is individual difference in susceptibility to exposure to these environmental risk factors (40, 45). These individual differences may originate from genetic variation between individuals.

Introduction to genetics

The human genome consists of deoxyribonucleic acid (DNA) that is usually a double-helix and has two strands running in opposite directions. Each strand is made up of nucleotides composed of a backbone with a sugar molecule (deoxyribose), a phosphate group and a base (51). There are four different types of bases; adenine (A), thymine (T), cytosine (C) and guanine (G). In the double-strand DNA, A always pairs with T (A-T) and G always pairs with C (G-C). The A-T base-pair has 2 hydrogen bonds and the G-C base-pair has 3 hydrogen bonds. Although more than 99% of all DNA is similar between individuals, certain base-pairs differ. These differences are called single nucleotide polymorphisms (SNPs) (Figure 2). Although there are millions of SNPs in human genome, only a minority of these variants is functional. Functional SNPs may alter gene expression (eQTL), protein structure or splice variants (52).

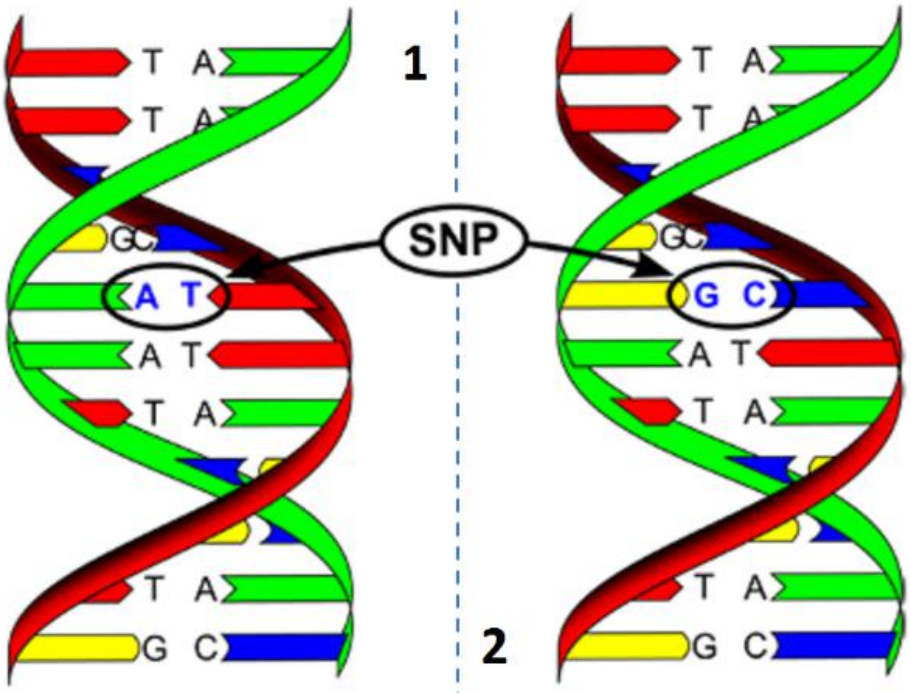

Figure 2. Single nucleotide polymorphism (SNP), a single base pair difference between individuals.

Genetic research designs

Candidate gene approaches have been used to investigate the association of SNPs in one gene or a few genes with the outcome of interest (53, 54). The selection of candidate genes is based on a-priori known biological function and is hypothesis driven. In contrast, genome-wide association (GWA) studies are hypothesis-free approaches testing hundreds of thousands genetic markers across the entire genome (55). However, most single GWA studies do not replicate consistently, which might be caused by environmental factors that trigger the development of symptoms or diseases only in subjects with a specific genotype (55, 56). More recently, hypothesis free genome-wide interaction (GWI) studies are used to identify genetic loci that affect the susceptibility for the effects of known harmful exposures on symptoms or diseases (57).

Genetic studies on respiratory symptoms

To date, only a few candidate gene studies, investigating associations between genetic variation and respiratory symptoms, have been performed. One of the studies showed that surfactant protein A (*SFTPA*) is a multifunctional protein that plays a role in protection of the lungs. Polymorphisms within *SFTPA* loci were associated with respiratory distress syndrome, wheeze and persistent cough in infants (58-60). There was a strong association between genetic variants at 17q21 near *ORMDL3* and wheeze in children (61). Polymorphisms in *IL-13* are associated with risk of recurrent wheeze (62). Savenije et al demonstrated that IL33-IL1RL1 pathway polymorphisms are associated with wheezing phenotypes and asthma (63). Tsai et al showed that polymorphisms in IL-13 were associated with increased risks of wheeze and asthma (64). Kim et al reported that β_2-adrenergic receptor polymorphisms were associated with nocturnal cough in atopic subjects but not atopy and bronchial hyperresponsiveness in a general population (65). Both polymorphisms in IL-4 Receptor α (*IL-4Rα*) and Toll-like receptor 4 (*TLR4*) were associated with work-related respiratory symptoms in bakery workers (66, 67). Dijkstra et al was the first to demonstrate that SNP rs6577641 was associated with chronic mucus hypersecretion (CHM) in the first GWA analysis on respiratory symptoms (68).

A respiratory symptom can be caused by different environmental exposures or can be a presentation of different underlying diseases with specific genetic or environmental origins. Susceptibility to these various exposures may be genetically determined and susceptibility loci may differ between exposures. The environment should thus be taken into account when studying the association between genetics and respiratory symptoms. So it is necessary to perform GWI studies to identify susceptibility genes interacted with risk environmental exposures on respiratory symptoms. As far as we know, no GWI study on respiratory symptoms has been performed or published so far. This may be because GWI studies are difficult to perform since both detailed phenotypic and

genotypic information should be acquired in addition to detailed and reliable exposure assessment (69). Cohorts for genome-wide association studies need to be large in order to have sufficient power to overcome the multiple testing penalty (Bonferroni corrected p-value = 0.05/number of tests). A genome wide interaction study requires an even larger number of subjects because subgroups of the population need to be analyzed, given the fact that not all subjects with genetic variant will have the exposure under study. Different strategies have been proposed to analyze the data with maximal power.

AIM OF THE THESIS

The aim of this thesis is to assess whether environmental exposures are associated with the level of lung function and respiratory symptoms, and to examine whether the effects of these exposures are dependent on genetic make-up.

STUDY POPULATIONS

In this thesis, we used three cohorts to conduct our research. The first cohort is a sample from the general population of Chinese Han children from Shantou, China. The other two cohorts are the LifeLines cohort study and the Vlagtwedde-Vlaardingen cohort study. These general population based cohorts both consist of exclusively Caucasian adult individuals of Dutch descent.

The Chinese Han children population

The Chinese Han children population included subjects from three areas in Shantou City, in the southeastern coast of Guangdong province, China (Figure 3). The first investigated area is Guiyu town that is one of the largest electronic waste (e-waste) destinations and recycling areas in the world. Guiyu is approximately 52km^2 and had a total population of 139,000 in 2009. Guiyu has received and handled 1.7 millions of tons of e-waste from overseas and domestic sources in 2007 according to the local government. More than 6000 small-scale family-run workshops (nearly 60% ~ 80% of families in the town) and 160,000 workers are

involved in the business of e-waste dismantling and recycling (70, 71). The second research area is Haojiang district located in the 31.6 km to the east of Guiyu. The third study area is Xiashan town located 5.8 km to the southeast of Guiyu. Haojiang and Xiashan are used as the reference areas without e-waste exposure. Preschool children, 3-8 years of age, were recruited from kindergartens in Guiyu, in Haojiang, and in Xiashan.

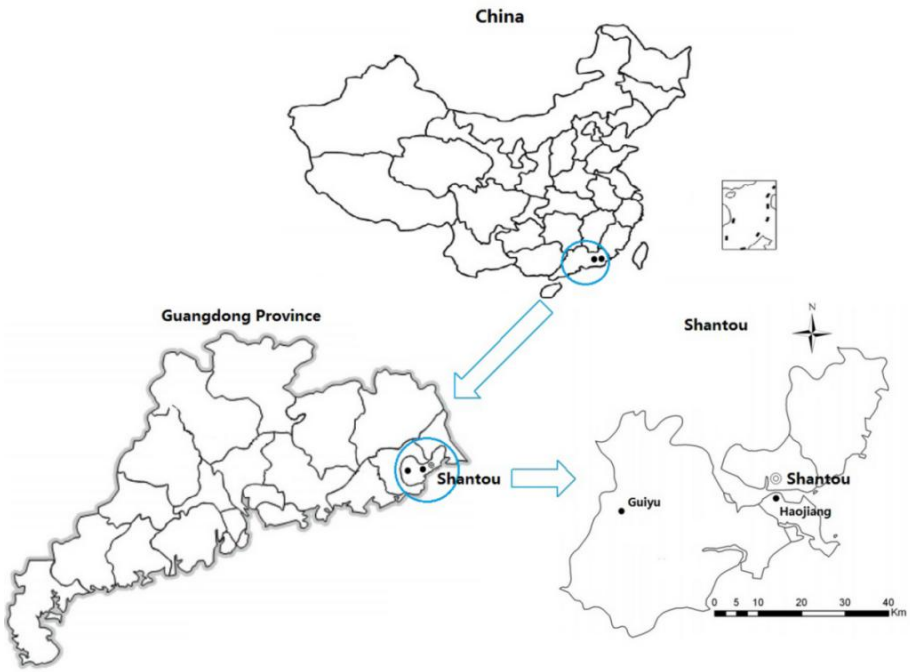

Figure 3. The location of study areas in Shantou, China (Guiyu, Haojiang and Xiashan).

The LifeLines cohort study

The LifeLines cohort is a large observational population based cohort study including subjects from the Northern provinces (Groningen, Friesland and Drenthe) of the Netherlands (Figure 4). The LifeLines cohort study was established in 2006 and included a total number of over 167,000 individuals which will be followed for 30 years. LifeLines is designed to investigate genes, exposures and their interactions in the etiology of complex diseases and healthy aging (72, 73). All subjects receive a baseline questionnaire and an invitation to a comprehensive health assessment, including spirometry, environmental exposure data, and presence of respiratory symptom information. Most of the work displayed in this thesis is performed on data from the first and second

data release of the LifeLines cohort study including approximately 13,300 unrelated adult individuals selected for genome-wide genotyping.

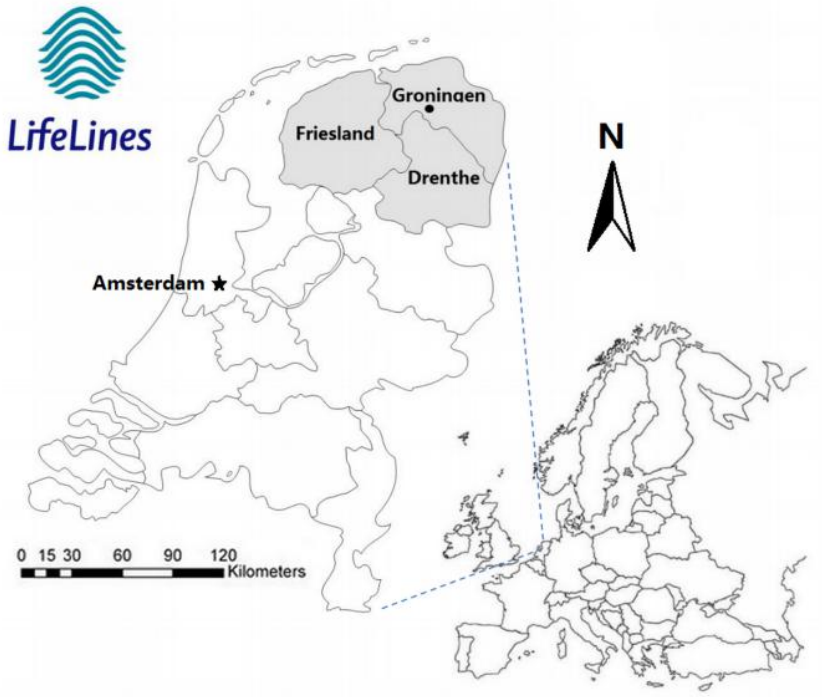

Figure 4. The location of study areas in the LifeLines cohort study (Groningen, Friesland and Drenthe) depicted grey.

The Vlagtwedde-Vlaardingen cohort study

The Vlagtwedde-Vlaardingen cohort is a general population-based cohort study including subjects from a rural area in the North-East of the Netherlands (Vlagtwedde) and subjects from an urban area in the South-West of the Netherlands (Vlaardingen) (Figure 5). The cohort started in 1965 and was followed for 25 years. The medical examinations were performed every 3 years until the last survey in 1989/1990. The study aimed to obtain knowledge on the prevalence of chronic respiratory symptoms and airway diseases as well as to gain deeper insight in determinants of these symptoms and diseases. Data were collected on endogenous factors such as age, sex, allergy and bronchial hyper-responsiveness, and exogenous factors such as tobacco smoking (10, 74). Genome-wide genotyping has been performed on blood samples from a subset of subjects included in the last survey (1989/1990). Genotypes are available for approximately 1,300 subjects.

Figure 5. The location of study areas in the Vlagtwedde-Vlaardingen cohort study (Vlagtwedde (a rural area) and Vlaardingen (an urban area)).

Outline of the thesis

In Chapter 2 we reviewed routes and adverse effects of e-waste exposure on several health outcomes including endocrine toxicity, respiratory system toxicity, developmental toxicity, reproduction toxicity, neurotoxicity, and genetic toxicity.

In Chapter 3 we reviewed associations of various heavy metal exposures with adverse effects on the health of children including respiratory system, cardiovascular system, nervous system, immune system, reproductive system, skeletal system and urinary system.

In Chapter 4 we assessed if living in e-waste exposed area was associated with blood concentrations of lead and cadmium, and with level of lung function in preschool children from China.

In Chapter 5 we performed a genome-wide association study to identify novel genetic loci associated with respiratory symptoms cough, dyspnea and phlegm.

In Chapter 6 we used a genome-wide interaction study to identify novel genetic loci that affect individual susceptibility to the effects of common occupational exposures on the respiratory symptoms cough, dyspnea and phlegm. Secondly we assessed whether the replicated loci were cis-acting expression quantitative trait loci (cis-eQTLs) in lung tissue.

In Chapter 7 we performed a genome-wide interaction study to identify novel genetic loci that affect individual susceptibility to ambient nitrogen dioxide exposure on the respiratory symptoms. Secondly we assessed whether the identified loci were cis-acting expression quantitative trait loci (cis-eQTLs) in lung tissue.

In Chapter 8 the results of these studies are summarized and discussed.

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CHAPTER 2

E-waste environmental contamination and harm to public health in China

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Abstract

The adverse effects of electronic waste (e-waste) on the human body have stirred up concern in recent years. China is one of the countries that confront serious pollution and human exposure of e-waste, and the majority of the population is exposed to potentially hazardous substances that are derived from informal e-waste recycling processes. This study reviews recent reports on human exposure to e-waste in China, with particular focus on exposure routes (e.g., inhalation and ingestion) and several toxicities of human (e.g., endocrine system, respiratory system, reproductive system, developmental toxicity, neurotoxicity, and genetic toxicity). Pieces of evidence that associate e-waste exposure with human health effects in China are assessed. The role of toxic heavy metals (e.g., Lead, cadmium, chromium, mercury, and nickel) and organic pollutants (e.g., polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyl (PCBs), polycyclic aromatic hydrocarbons (PAHs), polybrominated biphenyls (PBBs), polyhalogenated aromatic hydrocarbons (PHAHs), and bisphenol (BPA) on human health is also briefly discussed.

Keywords: e-waste; heavy metals; organic pollutants; hazardous; toxicity; human health; China

Introduction

Electronic-waste (e-waste) refers to end-of-life electronic products, including computers, mobile phones, washing machines, air conditioners, television sets, and others, which are composed of sophisticated blends of plastics, metals, among other materials. In recent years, e-waste pollution has been one of the most discussed global environmental issues. China (Guiyu and Taizhou), India (Delhi and Bengaluru), Nigeria (Lagos), and Ghana (Accra) are the most serious e-waste-contaminated countries, with little or no regulation for e-waste recycling or disposal [1]. About 80% of the e-waste is exported to Asia, and 90% of that is transported to China, which means that China is the largest destinations of e-waste from developed countries [2]. Meanwhile, China itself has generated considerable e-waste, which exceeds one million tons per year [3]. Hazardous chemicals can be discharged as a mixture from e-waste through irregular disposal or recycling processes, threatening the health of local residents [4]. This study compiles information from the literature about the effect of pollutants released from e-waste recycling areas of China on human health.

Environmental residue of pollutants from e-waste

Currently, the e-waste dismantling industry in China is mainly distributed among coastal areas such as Guiyu and Taizhou, which are crucial international e-waste dismantling base and key research areas. Heavy metals and organic pollutants are released into such medium as the atmosphere, soil, dust, and water, and residues of these harmful toxins are left in the environment. Specific e-waste-associated chemical elements and compounds exist in the form of components of the equipment, target product, and by-products during the recycling process. The main elements include lead, mercury, chromium, cadmium, zinc, copper, nickel, and other dozens of metals, mainly plastic components that contain polyvinyl chloride, polyethylene, acrylonitrile-butadiene-styrene polymers, polystyrene, polypropylene,

polyethylene terephthalate esters, brominated flame retardants, toner, surface coatings, and other harmful substances [5]. The risk of e-waste to the general population and surrounding environment steeply increases with the lack of guidelines of health and safety and improper recycling techniques, such as dumping, dismantling, inappropriate shredding, roasting, burning and acid leaching from complex components of e-waste and high-cost recycle (Fig. 1).

Fig.1 The informal e-waste recycling activities in Guiyu including roasting, burning, dismantling, acid leaching, inappropriate shredding, and dumping.

Studies show that a variety of heavy metals and organic pollutants have been detected in the scene of e-waste dismantling site and its surrounding environmental media (air, water, soil) of China. A survey conducted in Guiyu revealed elevated mean heavy metal concentrations in dust of workshop (Pb 110000, Cu 8360, Zn 4420, and Ni 1500 mg/kg) and in dust of adjacent roads (Pb 22600, Cu 6170, Zn 2370, and Ni 304 mg/kg). The Pb and Cu in road dust from e-waste site were 330, 106, and

371, 155 times higher than non-e-waste sites located 8 and 30 km away, respectively [6]. Another study evaluated the effect of e-waste recycling activities in Guiyu. Heavy metal concentrations in surface water and sediment samples from Lianjiang River decreased from Guiyu (upstream) to Haimen Bay (downstream, the estuary), Cu concentrations in surface water of Guiyu were 2.4 to 131 times higher than the reference Cu background concentration of water, and Cu concentrations in sediment samples from Guiyu were 3.2 to 429 times larger than those from the reference background [7]. Total concentration of 13 polybrominated diphenyl ethers (PBDEs) in atmosphere from an e-waste dismantling area in Taizhou was 506 pg/m^3 in summer and 1662 pg/m^3 in winter, which was 7 times higher than that of the reference urban region [8]. Total polycyclic aromatic hydrocarbons in soil from an e-waste recycling site in Taizhou ranged from 371.8μg/kg to 1231.2 μg/kg, and total polychlorinated biphenyl (PCB) concentrations ranged from 52.0μg/kg to 5789.5 μg/kg, which were 2.1 to 232.5 times higher than that from the reference site [9]. Thus, mixture pollutants will release to environmental media from informal e-waste recycling activities.

Human exposure to heavy metals and organic pollutants derived from e-waste

Exposure routes can vary dependent on the substance and recycling process. Humans can become exposed to the hazardous components of e-waste in air, soil, dust, water, and food sources through several routes, including ingestion, inhalation, and dermal absorption (Table 1). To date, surveys of pollution level of the human body were mostly concentrated in the Guiyu area. A long-term study conducted by our research team in Guiyu showed that 81.8%, 70.8%, 69.9% blood lead level of local children exceeded the limit (10 μg/dL) in 2004, 2006, and 2008, respectively [4,10,11]. Median umbilical cord blood chromium levels of neonates from Guiyu in 2006 and 2007 were 93.89 and 70.60 μg/L, respectively, whereas the normal range of blood chromium is 0μg/L to 0.2μg/L [12]. From 2004 to 2007, the median cord blood and mean placental cadmium

(3.61 μg/L and 0.17 μg/g, respectively) from Guiyu was higher than those from the reference (1.25 μg/L and 0.10 μg/g, respectively) [13]. The informal e-waste recycling not only leads to serious heavy metal pollution but also causes grievous organic pollution, which severely affects the health and daily life of local residents. Concentrations of PBDEs and dechlorane plus (DP) in serum from local residents of Guiyu were measured, and the result showed that the median concentrations of total PBDE was three times higher than that of the reference group. The DP concentration from Guiyu (median, interquartile range; 42.6 ng/g, 7.8 to 465ng/g lipid) was larger than that from reference (median, interquartile range; 13.7 ng/g, 0.93 to 50.5ng/g lipid) [14,15]. A study conducted to explore the burdens of polybrominated biphenyls (PBBs), PBDEs, and PCBs among cancer patients from an e-waste disassembly area in Taizhou showed that BDE-47 was the most predominant PBDE congeners, and concentrations of PCB were the highest among the three subfamilies of polyhalogenated aromatic hydrocarbons measured, suggesting that a high cancer incidence in the e-waste disassembly sites may be related to the higher burdens of PBBs, PBDEs, and PCBs in tissues of local residents [16]. Toxic substances released from e-waste may enrich through animals and plants, and finally enter the human body through the food chain, which will definitely harm the health of local populations.

Health effects caused by e-waste pollution

Pollutants that stay in the human body can damage multiple systems, such as endocrine, respiratory, nervous, reproductive system, with a genetic toxicity (Table 1). For instance, lead can accumulate long term in the environment and then affect multiple systems, especially the nervous system, blood, and digestive system. Cadmium can also accumulate long term in the multiple organs, such as kidney and liver, with apparent chronic toxicity. Inorganic mercury tends to transform into methylmercury under the action of microorganisms, and this action leads to the enrichment of mercury in the food chains. Consequently, coastal

populations with traditionally high dietary intake of freshwater fish or seafood have the highest dietary exposure to mercury, and low dose of mercury may affect the nervous system of adults [17]. Chromium mainly exists in the form of trivalent and hexavalent chromium in nature. The toxicity of hexavalent chromium is the largest toxicity among many valence of chromium, which can cause respiratory inflammation and even mutation or canceration of cells caused by DNA damage. PBDEs with high durability and lipophilicity are used for the flame-retardant additives in plastics, electrical appliances, and television sets. PBDEs are easy to accumulate in the body fat that they can cause adverse effects on neural development and disrupt endocrine function. Dioxins are a group of chlorinated or brominated organic chemicals or a mix of them, and the main form usually includes chlorinated dioxins (PCDD/F/Bs), brominated dioxins (BBDD/F/Bs), and mixed bromo-chloro dioxins (PXDD/F/Bs). They are found in human carcinogens and can cause noncancerous effects such as atherosclerosis, hypertension, and diabetes. Long-term exposures to dioxins will lead to the disruption of the nervous, immune, reproductive, and endocrine system [18].

Table 1 Classification, sources and routes of e-waste components.

Pollutant categories	Health effects and potential toxic mechanism	Commercial sources of exposure	Ecological source of exposure	Route of exposure
Persistent organi pollutants				
Polybrominated diphenyl ethers (PBDEs)	Carcinogenic, genotoxic, endocrine disrupting, metabolic syndromes, low birth weight, decrease in IQ, decreased psychomotor and mental development, neurobehavioral development, decreased female fertility	Fire retardants for electronic equipment	Air, dust, food, water, soil, and food	Ingestion, inhalation, and transplacental
Polychlorinated biphenyls (PCBs)	Probably carcinogenic, thyroid function, cognitive function and development, neuropsychological development, attention deficits, decrease in IQ, intellectual impairment, low birth weight and reduced growth	Dielectric fluids, lubricants and coolants in generators, capacitors and transformers, fluorescent lighting, ceiling fans, dishwashers, and electric motors	Air, dust, soil, and food (bioaccumulative in fish and seafood)	Ingestion, inhalation or dermal contact, and transplacental
Polychlorinated dibenzodioxins and dibenzofurans (PCDD/PCDFs)	Carcinogenic, thyroid function, type II diabetes, obesity, cardiovascular disease, fetal toxicity, reproductive toxicity, feminization, changes in sex ratio at birth, and infantile autism	Released as a combustion byproduct but also found in dielectric fluids, lubricants and coolants in generators,	Air, dust, soil, food, water, and vapour	Ingestion, inhalation, dermal contact, and transplacental

		capacitors and transformers, fluorescent lighting, ceiling fans, dishwashers, and electric motors		
Polycyclic Aromatic Hydrocarbons (PAHs)	Carcinogenic, mutagenic, teratogenic, toxicity of development and reproduction, and DNA damage, lung, skin and bladder, potentially larynx and kidney	Released as combustion byproduct	Released as combustion byproduct, air, dust, soil, and food	Ingestion, inhalation, and dermal contact
Elements				
Lead (Pb)	Impact enzyme activity, membrane ion channel, signal molecules, gene expression, neurogenesis, and neurodegeneration, decrease in IQ, impaired cognitive function, anemia, neuropsychological function, nephrotoxic, adult hearing loss	Printed circuit boards, cathode ray tubes, light bulbs, televisions, and batteries	Air, dust, water, and soil	Inhalation, ingestion, and dermal contact
Cadmium (Cd)	Affect enzyme activity, kidney injury, decreased bone density, lung damage, carcinogenic, mutagenetic, decrease in IQ, neurodevelopment, adult hearing loss, low birth weight, cell apoptosis, and gene expression	Switches, springs, connectors, printed circuit boards, batteries, infrared detectors, semi-conductor chips, ink or toner photocopying machines, cathode ray tubes, and mobile	Air, dust, soil, water, and food (especially rice and vegetables)	Inhalation and ingestion

		phones		
Mercury (Hg)	Induction of oxidative stress, inflammatory, and nerve cell apoptosis, affect enzyme activity and genetic modification, nephrotoxic, memory loss, immune system toxicity, decrease in IQ, genotoxic, decreased fertility	Thermostats, sensors, monitors, cells, printed circuit boards, and cold cathode fluorescent lamps	Air, vapour, water, soil, and food (bioaccumulative in fish)	Inhalation, ingestion, and dermal contact
Chromium (Cr)	Carcinogenic, genotoxic, mutagenetic, ovotoxic, lung function, allergic reaction and contact dermatitis, glycometabolism, reproductive developmental anomalies	Printed circuit boards, cathode ray tubes, light bulbs, televisions, pigment, and batteries	Air, dust, water, and soil	Inhalation and ingestion

Endocrine toxicity

Most environmental endocrine disruptors have estrogen-like effects, and these pollutants interfere with the endocrine function of animals to produce acute or potential toxic effects. Xenobiotics, such as PBDEs, PCDDs, and PCDFs, can be combined with hormone receptor in the organism, affecting the synthesis, secretion, transport, metabolism, combination of hormones and interfering with normal hormone levels in the human body to disrupt the endocrine system. Endocrine disturbances are associated with various human diseases, such as obesity, heart disease, diabetes, infertility, cancer and neurological defects. Several studies show that e-waste contaminant exposure interferes with the balance of thyroid hormones and sex hormones in the local population. The reported effects on thyroid-stimulation hormone (TSH) are inconsistent, with some studies reporting higher TSH levels in the exposed populations [19,20] and other studies indicating lower TSH levels with e-waste exposure [21-24]. Tetraiodothyronine (T4) levels in the exposed population were not higher than those of the reference population. Thus, an inverse relationship existed between PBDEs and T4 [19,21-23]. Given that different findings may be caused by insufficient sample sizes (most studies with sample sizes less than 100 persons), studies with larger sample size and control of confounding factors are required to accurately investigate the effect of e-waste on thyroid function. Meanwhile, a study conducted to 187 man in an e-waste dismantling area in China found that the levels of male's hormone, such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), were higher than those in the reference group, and testosterone was lower than that in the reference group in male population aged 31 to 60 [25]. Similarly, the levels of sex hormones estradiol and progesterone were disrupted in pregnant women from the e-waste recycling site [26]. Therefore, the organic toxicity from e-waste on the endocrine system of the body should be focused on. Although the above-mentioned studies reveal that e-waste pollution has influenced the levels of thyroid and sex hormone, its mechanism is unclear at present and still requires further study.

Respiratory system toxicity

Harmful substances mainly enter the human body through respiratory stimulation, airway inflammation, and lung function damage. Particles can stimulate macrophages to produce reactive oxygen species and inflammation medium, and this action results in injury of trachea and lung tissue, thereby affecting the elasticity of the lung tissue and the respiratory function. However, the precise mechanism of lung damage caused by particles is unclear [27]. Associations between exposure to metals (Cr, Mn, and Ni) and lung function were examined in primary school children from our previous survey in Guiyu and a control town. Living in Guiyu was associated with a decreased forced vital capacity (FVC) in 8- to 9-year-old boys. Height-adjusted multivariate regression analysis showed significant negative associations between blood chromium levels and FVC in 11- and 13-year-olds and serum nickel levels and FVC in 10-year-olds [28]. Children who breathe in polluted air for a long time, especially for children in the stage of rapid development, will experience lung function decline.

Developmental toxicity

The fetal period is the key period of the growth and development, and extremely sensitive to the toxic substance such as heavy metal and organic pollutants. Our previous studies demonstrated that heavy metals and organic pollutant from e-waste were associated with stillbirth, premature delivery, and anencephaly, and the neonatal Apgar score, height and weight were lower than those of children from the reference area [29-33]. Growth hormones, such as placental insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-binding protein-3 (IGFBP-3) from an e-waste exposed area, were higher than those from the reference group. This result shows that IGF-1 and IGFBP-3 can be used as the monitoring indicators of fetal intrauterine growth and development [34]. Children are susceptible to environmental pollution and their normal growth and development are disturbed when they are exposed to harmful substances. Lead inhibits the synthesis and utilization of hormones such

as thyroid stimulating hormone (TSH) and insulin-like growth factor (IGF), in stunting physical development of children by disturbing the absorbing of calcium, iron, and zinc. A negative correlation was found among height, weight and child blood lead in Guiyu, and exposure to manganese or nickel will reduce height, weight, and body mass index of local children [35]. The weight and chest circumference were larger from the high chromium group than those from low chromium group [36]. Handling and disposal of unwanted electronic products is frequently unsafe and unregulated. Thus, extensive e-waste dismantling and recycling activities pose a potential risk and an expanding challenge to the growth and development of local neonates and children.

Reproduction toxicity

The frequent occurrence of reproductive disease has stirred up wide public concern on environmental pollution. A study found that estrogen-like effect of bisphenol A (BPA) may cause reproductive toxicity to the body [37]. Specifically, BPA can cause female precocious puberty and reproductive system tumor to affect fertility, and it can reduce sperm quality and quantity. Outpatient data were collected from a Guiyu hospital and a reference hospital from 2001 to 2009. The statistical results show that the incidence of male reproductive disease was 0.753‰ and 0.355‰ in 2004 and 2009, respectively. The incidence of azoospermia, asthenozoospermia, and epididymitis from Guiyu hospital was higher than those from the reference hospital [38]. The results indicate that male reproductive health may be affected by environmental pollutants, and this finding requires confirmation in further epidemiological investigation.

Neurotoxicity

A study from China found that every 10 μg/L increase of blood Pb concentration is associated with a deficit of 0.71 IQ points [39]. A neonatal behavioral neurological assessment (NBNA) study conducted in Guiyu by our group showed that neonatal exposure to high lead was associated with low NBNA scores. The temperament questionnaires for 3- to

7-year-old children were used to assess the temperament of children, and results indicated that Guiyu children had higher activity level, approach-withdrawal, and adaptability than those from the reference group. Blood lead levels among Guiyu children were much higher than those in children from the reference group, which demonstrated that high lead load influenced the temperament of children, while temperament characteristics are associated with some intelligence area in the development of the intelligence of children [40]. Thus, high lead load affects the intellectual, psychological, and behavioral health of children.

Genetic toxicity

Hazardous materials released from e-waste dismantling process could damage human health and even pose toxic effects to genetic materials, resulting in DNA damage, gene expression, and alteration of chromosome structure.

DNA damage

Oxidative damage is an important aspect among several DNA damage mechanisms. It reflects an imbalance between the systemic manifestation of reactive oxygen species and the ability of a biological system to readily detoxify the reactive intermediates or to repair the resulting damage, with an imbalance in the oxidant/anti-oxidant system [41]. Heavy metal can induce the body to produce a large number of free radicals that contribute to oxidative stress and destroy the metabolic balance of oxygen-free radicals found in the body. A Chinese study showed that the levels of reactive oxygen species in white blood cell from the residents of Guiyu were higher than those from the reference group [42]. Another study demonstrated that super oxide dismutase and glutathione peroxidase from Guiyu pregnant women were lower than those from the reference group, while malondialdehyde from Guiyu pregnant women were higher than that from the reference group [26]. The DNA injury rate and tail length of lymphocyte in neonate from Guiyu were higher than those from the reference group, and positive correlations were found between blood

chromium and DNA injury rate and tail length of lymphocytes. This result implies that exposure to chromium can lead to DNA and cell damage [12]. The frequency distribution of the aminolevulinic acid dehydratase type 2 allele in newborns from Guiyu was lower than that of the reference group [43,44]. Other studies also indicated that chromosome aberration rate, micronucleus rate, lymphocyte percentage of tail DNA, lymphocyte lengths of tail, and lymphocyte olive lengths of tail from Guiyu were higher than those from the reference group [43,44]. Telomere of DNA shortening may cause adverse birth outcomes and increase cancer risk. Placental cadmium was also negatively correlated with telomere length from Guiyu samples based on a previous study [45].

Alterations in gene expression

MicroRNA (miRNA) is a small non-coding RNA molecule (containing about 22 nucleotides), which functions in RNA silencing and post-transcriptional regulation of expression, modification, translation, transcription of gene. It is important in the response process of cell to environment. A study from China obtained miRNAs expression profiling of male from Guiyu and reference group, and 109 downregulated and 73 upregulated expression miRNAs were found based on the comparison of the two groups of miRNAs expression profiling [46]. Mother placental cadmium levels and metallothionein (MT) from Guiyu were higher than those from the reference group, and calcium binding protein S100P level of Guiyu was lower than that from the reference group. The placental cadmium was negatively correlated with S100P and positively correlated with MT [47]. Another study found that the positive expression rate of MT in placenta tissue from Guiyu was 67.0%, which is significantly higher than that in placenta tissue from reference group (32.7%). The expression quantity of MT in placenta was positively correlated with placental cadmium levels and umbilical cord cadmium levels [13]. Therefore, the biological activity of expression of S100P and MT in the placenta is likely to be affected upon exposure to heavy metals.

Alterations of chromosome structure

Telomere is a terminal structure of eukaryotic chromosomes. It is composed of hundreds of thousands of highly repetitive tandem repeats (TTAGGG in human) and associated proteins. Telomere serves as a cap on the end of chromosomes to maintain integrity of the chromosomes and stability of the genome, and to prevent end-to-end chromosomal fusion [48]. Telomere is important in placental development and maturation. Shortened placental telomere is believed to cause adverse birth outcomes, such as fetal growth restriction and low birth weight [49, 50]. Shortened telomere is also related to increased cancer risk and aging [51-54]. One of our previous studies found that placental telomere length was negatively correlated with placental cadmium concentration. Placental cadmium concentration of 0.0294 μg/g may be a critical point at which attrition of placental telomere commenced. Exposure to cadmium pollution during pregnancy may be a risk factor for shortened placental telomere length [45]. Several biomarkers, such as monomer, fragments, translocation, satellite, quadriradials, total aberrations, and micronuclear rates, were used to perform chromosomal aberrations and cytokinesis blocking for each subject in a study conducted in Tianjin, China. The total chromosome aberration rates (5.50%) and micronuclear rates (16.99%) of the exposure group were higher than those of the reference group, and the chromosome aberration and micronucleus rate were higher in women than those in men [55].

Perspectives

E-waste pollution of China adversely affects the health of dismantling workers and nearby residents based on the above-mentioned literature. The research results from various studies are inconsistent, which may be caused by lack of sufficient sample size of the studies and inappropriate adjustment for confounding factors. Most of the studies are descriptive, with usual information about exposure collected at the same time as the health effects are measured in a group of individuals during a specific period. Thus, identifying clear temporal relations between exposures and health effects is difficult. By contrast, cohort studies are generally suitable in exploring the association between e-waste exposures and health effects. The types and compositions of e-waste are diverse and complex and may give joint toxicity on the human body rather than single e-waste pollutant damage. Most of current research focuses on the toxic effect of a certain toxic substance. Thus, future studies should pay more attention to the joint toxicity of a variety of toxic substances from e-waste. Multinational and multi-region research on the health effect of e-waste should be conducted to obtain accurate scientific conclusions.

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Compliance with ethics guidelines

Xijin Xu, Xiang Zeng, H. Marike Boezen, and Xia Huo declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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CHAPTER 3

Children with health impairments by heavy metals in an e-waste recycling area

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Abstract

E-waste recycling has become a global environmental health issue. Pernicious chemicals escape into the environment due to informal and nonstandard e-waste recycling activities involving manual dismantling, open burning to recover heavy metals and open dumping of residual fractions. Heavy metals derived from electronic waste (e-waste), such as, lead (Pb), cadmium (Cd), chromium (Cr), manganese (Mn), nickel (Ni), mercury (Hg), arsenic (As), copper (Cu), zinc (Zn), aluminum (Al) and cobalt (Co), differ in their chemical composition, reaction properties, distribution, metabolism, excretion and biological transmission. Our previous studies showed that heavy metal exposure have adverse effects on children's health including lower birth weight, lower anogenital distance, lower Apgar scores, lower current weight, lower lung function, lower hepatitis B surface antibody levels, higher prevalence of attention-deficit/hyperactivity disorder, and higher DNA and chromosome damage. Heavy metals influence a number of systems and organs, resulting in both acute and chronic effects on children's health, ranging from minor upper respiratory irritation to chronic respiratory, cardiovascular, nervous, urinary and reproductive disease, as well as aggravation of pre-existing symptoms and disease. These effects of heavy metals on children's health are briefly discussed.

Keywords: Electronic waste; Heavy metals; Exposure; Children; Guiyu

Introduction

Electronic waste (e-waste), such as waste laptop, and desktop computers, monitors, cell phones, keyboards, printers, copiers, televisions, refrigerators, and washing machines, are the endpoint of the vast amounts of electronic items in modern society. It was estimated that global e-waste generation was 41.8 million tonnes in 2014 and may increase to 65.4 million tonnes by 2017 (1,2). E-waste contains heavy metals that can be released during inappropriate recycling processes and disposal, resulting in harming humans, animals, vegetation or other environmental materials (3-6). The countries most affected by informal e-recycling are China (Guiyu and Taizhou), India (Bengaluru and Delhi) and some African countries (Lagos in Nigeria, Accra in Ghana), where e-waste has been recycled or disposed with little or no regulation, using less advanced technology (7-11). Humans can become exposed to heavy metals in air, soil, dust, water, and food sources through several routes that include ingestion, inhalation, and dermal absorption from combustion, discharges and manufacturing facilities (12,13). Heavy metals cannot be degraded into less hazardous end products, which is different from organic pollutants with biodegradable capability. Although segmental heavy metals such as trace elements are essential to maintain normal metabolism to a small extent, most of heavy metals have a potentially adverse effect on human health when their concentrations are exceeded the tolerance levels due to the bio-accumulation of e-waste components and by-products in living organisms over time (14).

Children are considered more susceptible to hazardous metal substances compared to adults for several reasons: excess routes of exposure (breastfeeding, placental exposures, hand-to-mouth, object-to-mouth activities); Children have higher basal metabolic rate compare to adults, and they have higher comparative uptakes of food and lower toxin elimination rates; children have higher ventilation per minute in relation to body size compared to adults, and they can inhale more harmful metal

substances: children have a much larger surface area in relation to body weight than adults, and they can load higher amount of toxicants in their body through dermal absorption; their organs or tissues are development and thus more sensitive to perturbed cellular process; children have often higher physical activity interacted with environment compared to adults, and they are likely to receive bigger doses of toxicants, relative to their size, than adults; some exposure such as ETS and e-waste in the home is difficult to avoid for children (15). In addition, children from Guiyu are forced to exposure to the full of e-waste pollution of environment from home, school, and play areas located near landfills and e-recycling business. It is worth mentioning that the children of e-waste recycling workers also confront take-home contamination from their parents' clothes and persons, and in particular from firsthand high-level exposure if recycling are performed in their homes. One study investigated the levels of heavy metals in workers and local adult residents in Taizhou (an e-waste exposed area in Zhejiang province, China). This study showed that the blood lead levels of worker and local adult residents were 15.1 and 8.4 μg/dL, respectively (16). One of our previous study reported that the blood lead levels of children in Guiyu (an e-waste exposed area in Guangdong province, China) was 7.3 μg/dL (17). According to these two study results, adults seem to be more exposure to the e-waste pollution, but there is little difference in e-waste exposure between children and adults. The truth is not to be ignored that children are more easily damaged than adults when confronting even the same levels of environmental pollution because their immature systems are unable to handle and excrete some toxic materials efficiently, and their poor self-protection sense and ability against environmental pollution make them hard to avoid damage resulted from e-waste pollution.

Guiyu, has a 30-year e-waste dismantling history, is one of the largest e-waste destinations and recycling areas in the world. Nearly 60-80% of families in Guiyu have engaged in e-waste recycling operations run by small scale family workshops (18). About 60 chemical elements can be found in e-waste, such as lead, cadmium, chromium, manganese, nickel,

mercury, copper, arsenic, zinc, iron, and aluminum, many of which are potentially, or known to be, hazardous (2,19). These metals are used in products such as circuit boards, semi-conductor chips, cathode ray tubes, coatings and batteries (10). E-waste-derived heavy metal pollution is mainly from several e-waste recycling activities including roasting, burning, acid leaching, inappropriate shredding and dismantling (20). Informal and hazardous e-waste recycling activities caused local environmental pollution which poses a threat to the human health of local residents, particularly in children (Fig. 1). Heavy metal pollution above the permissible limits has been found in Guiyu according to our previous studies (17,18,20-22). In this review, we provided the first assessment of heavy metals on children of Guiyu, and survey literatures to provide updated information about association between heavy metals from e-waste and respiratory, cardiovascular, nervous, reproductive, and urinary toxicity in children, and preventative measures to reduce exposure.

Fig. 1. The scene of the e-waste recycling in Guiyu, China.

Routes of exposure

Routes of exposure can vary depending on the substance and e-waste recycling process involved (Table 1). In general, exposure to the heavy metals of e-waste can happen through inhalation, ingestion and dermal absorption. Heavy metals are primarily absorbed through respiration in terms of dust, aerosols or vapor. Children may also contact and ingest heavy metals by means of mouth and skin. A lot of heavy metals are

pernicious because they incline to bio-accumulate in human body. Compounds accumulate in organisms when they are taken in and stored faster than they are broken down (metabolized) or excreted. In addition to occupational exposure, people can contact with e-waste materials and related pollutants mainly through environmental contact with soil, dust, air, water, and food sources. The most significant input of heavy metals to surface environment in various e-waste processing activities is probably due to the recycling of printed circuit boards and other electronic devices laden with heavy metals (9,23). The recycling procedure involves melting of solder from circuit boards over makeshift coal grills in order to sort chips, capacitors, and diodes that are then sold to electrical appliance factories. Exposed to high levels of heavy metal pollutants can result in acute and chronic toxicity, such as damage to the central and peripheral nervous systems, blood composition, lungs, kidneys, liver, and even death. On the one hand, heavy metals assimilate into the body through the blood circulation system and become enriched different organs; On the other hand, heavy metals can combine with protein molecules, such as binding of cadmium to sulfhydryl groups in proteins to inhibit enzyme activity, therefore undermining the normal physiological and biochemical reactions of human health (24).

60 **Table 1.** Health effects of exposure to heavy metals from e-waste contributed by our laboratory.

Characteristic	Setting	Population	Toxicant	Health effect
Lung function				
Zheng et al	Exposed vs reference	Children (aged 8–13 years; n=144)	Chromium, manganese, and nickel	Blood manganese: 20.6 vs 14.9 μg/L, $p < 0.01$. Nickel: 5.3 vs 3.0 μg/L, $p < 0.01$. FVC in boys aged 8–9 years: 1859 vs 2121 mL, $p = 0.03$. Decrease in FVC with increased chromium (11-year-old and 13-year-old), decreased FVC with increased nickel (10-year-olds)
Nervous				
Liu et al	Exposed vs reference	Preschool (aged 3–7 years; n=303)	Lead	Blood lead: 13.2 vs 8.3 μg/dL, $p < 0.01$. Temperament scores: activity level (mean ± SD 4.53 ± 0.83 vs 4.18 ± 0.81), adaptability (4.96 ± 0.73 vs 4.67 ± 0.83) and approach-withdrawal (4.62 ± 0.85 vs 4.3 ± 0.89)
Liu et al	Exposed	Preschool (aged 3–7 years; n=240)	Lead, manganese, and cadmium	Blood lead: 7.33 μg/dL (5.91-9.13), blood cadmium: 0.69 μg/L (0.54-0.92), blood manganese: 17.98 μg/L (15.31-21.77). The serum S100β was negatively associated with inattention and impulsivity-hyperactivity based on the CTRS in the low lead group, while positively associated with ADHD, DSM-IV hyperactivity/impulsivity, inattention, hyperactivity index, and Rutter antisocial behavior of the CTRS.
Li et al	Exposed vs reference	Neonate (younger than 1 month; n=152)	Lead	Cord blood lead: 113.3 vs 60.4 μg/L, $p < 0.001$; meconium lead: 2.5 vs 1.2 μg/g, $p < 0.001$. NBNA scores: total (38.46 vs 38.92, $p = 0.043$), behaviour cluster (10·91 vs 11.29, $p = 0.012$). Negative associations between meconium lead and total NMNA ($r = –0.903$, $p < 0.01$), activity tone ($r = –0.637$, $p < 0.01$), and behaviour ($r = –0.826$, $p < 0.01$) scores

Reproductive				
Xu et al	Exposed vs reference	Neonate (younger than 1 month; n=531)	Lead	Cord blood lead: 10.78 vs 2.25 μg/dL ($p < 0.01$), correlated with recycling activity. Higher rates of adverse birth outcomes: stillbirth (4.72% vs 1.03%, $p < 0.05$), preterm (5.68% vs 5.24%, $p < 0.05$), lower birthweight (3168 vs 3258 g, $p < 0.05$), and lower Apgar scores (9.6 vs 9.9, $p < 0.05$)
Skeletal				
Yang et al	Informal recycling	Preschool (aged 3–8 years; n=246)	Lead, cadmium	Blood lead (BLLs): 7.30 μg/dL; Blood cadmium (BCLs): 0.69 μg/L. The average of BCLs increased with age. BLLs were negatively correlated with both height and weight, and positively correlated with bone resorption biomarkers
Growth				
Zheng et al	Exposed vs reference	Children (aged 8–13 years; n=144)	Manganese and nickel	Height: 126.8 vs 135.0 cm, $p < 0.001$. Weight: 24.7 vs 30.2 kg, $p < 0.01$. BMI: 15.2 vs 16.5, $p < 0.01$. Negative correlations between serum manganese and height ($r_s = –0.303$, $p < 0.001$) and weight ($r_s = –0.228$, $p = 0.006$) and serum nickel and height ($r_s = –0.417$, $p < 0.001$), weight ($r_s = –0.399$, $p < 0.001$), and BMI ($r_s = –0.213$, $p = 0.011$)
Zheng et al	Exposed vs reference	Preschool (aged 1–7 years; n=278)	Lead and cadmium	Blood lead: 13.17 vs 10.04 μg/dL ($p < 0.01$); blood cadmium:1.58 vs 0.97 μg/L ($p < 0.01$). The average of BLLs increased with age in Guiyu ($p < 0.01$). Mean height of children in Guiyu is significantly lower than that in Chendian ($p < 0.01$)
Huo et al	Exposed vs reference	Preschool (younger than 6 years; n=226)	Lead	Blood lead: 15.3 vs 9.94 μg/dL ($p < 0.01$). No differences in height, weight, chest circumference, or head circumference

62	DNA damage					
	Li et al	Exposed vs reference	Neonate than 1 n=302)	(younger month;	Chromium	Comet assay: DNA damage (33.2% vs 10.7%, $p < 0.01$), length of tail (4.49 ± 1.92 vs 2.09 ± 0.65 µm, $p < 0.01$). Blood chromium correlated with DNA damage ($r_s = 0.95$, $p < 0.01$) and tail length ($r_s = 0.95$, $p < 0.01$)

Human health effects

Sporadic heavy metal pollution events have occurred, like the historic Minamata mercury pollution in 1953, or Jinzu River cadmium contamination in 1955, both in Japan, enabling a number of short and long term epidemiological studies to determine the effects of heavy metal exposure on human health. The kinds and doses of heavy metal exposures can lead to diverse impacts on human health, especially child health. For instance, lead exposure can result in the production of autoantibodies against neural proteins, including myelin basic protein and glial fibrillary acidic protein, deducing that lead can aggravate neurological disease by increasing the immunogenicity of nervous system proteins (25). The first sign of the renal lesion due to cadmium exposure is usually a tubular dysfunction which progress to more severe kidney damage, evidenced by an increased excretion of low molecular weight proteins or enzymes, and a decreased glomerular filtration rate (GFR) (26,27). The toxicity of methyl mercury (MeHg) to the developing brain is recognized in several studies, such as effects on neurotransmitter systems, induction of oxidative stress and disruption of microtubules and intracellular calcium homeostasis. Moreover, In vitro data show that very low levels of MeHg can inhibit neuronal differentiation of neural stem cells (28-30). Children living in Guiyu have significantly higher blood chromium levels (BCr) compared with those of Chendian during the period from 2004 to 2008 ($p < 0.001$). Weight and chest circumferences are significant differences between high- and low-exposure groups according to our previous study ($p < 0.01$) (31). Our previous study demonstrated that cadmium pollution was associated with short placental telomere in Guiyu (32). In addition, several susceptibility factors such as age, nutritional status and predisposing conditions can disrupt the impact of heavy metals on humans to some degree. Epidemiological and animal model data indicate that body systems affected by heavy metal exposure are the respiratory, cardiovascular, nervous systems, immune, skeleton, and urinary systems. The exposure to other toxicants such as persistent organic pollutants (POPs) are needs to be examined with heavy metal

pollutants in future studies to accurately assess their risk to human health.

Respiratory system

Multitudinous studies conclude that several types of heavy metals can both affect the airways at high concentration or long-term exposure to lower concentrations. Numerous findings show heavy metals from e-waste exposure may entail detrimental respiratory consequences in children. Symptoms or diseases, such as nose and throat irritation, followed by cough, wheezing and dyspnea or more seriously result in asthma, usually occur after exposure to increased levels of certain heavy metals such as lead, cadmium, chromium, manganese, nickel, arsenic, mercury, cobalt or vanadium. Elevated blood manganese levels and increased serum nickel levels are observed in primary school children in the informal e-waste recycling town of Guiyu compared to their peers in Liangying town without any history of e-waste recycling. Using forced vital capacity (FVC) as a marker for lung function, boys aged 8-9 years with increased levels of manganese and nickel in Guiyu have significantly lower lung function than that of the reference (33). Razi et al. demonstrate that elevated levels of serum lead and mercury, and low levels of zinc and selenium, may have disturbed the antioxidant system in children with recurrent wheezing (34). Lead exposure and its blood concentration in children have been shown to directly relate to immunoglobulin E (IgE) production which is interrelated with asthma in both humans (35-37) and rodent models (38,39). Smith et al. show that asthmatic children are over 5 times more likely to have elevated blood lead levels (EBLLs) than non-asthmatics (40).

Cardiovascular system

Population studies demonstrate a link between heavy metal exposure and subsequent development of hypertension, endothelial injury or dysfunction, arteriosclerosis, and cardiovascular disease. In vivo and in vitro studies show that chronic heavy metal exposures, such as lead,

cadmium, aluminum, mercury and arsenic, cause hypertension and cardiovascular disease by a series of cascade reactions promoting oxidative stress (41-43), impairing nitric oxide signaling (44), augmenting adrenergic activity (45,46), increasing endothelin production (47), altering the renin-angiotensin system (48,49), raising vasoconstrictor prostaglandins and lowering vasodilator prostaglandins (47,50,51), promoting inflammation, disturbing vascular smooth muscle Ca^{2+} signaling (52), diminishing endothelium-dependent vasorelaxation and modifying the vascular response to vasoactive agonists (53,54). Moreover, several heavy metals have been shown to cause endothelial injury (55,56), impede endothelial repair (55-57), inhibit angiogenesis (58,59), reduce endothelial cell growth (60,61), suppress proteoglycan production, stimulate vascular smooth muscle cell proliferation and phenotypic transformation, reduce tissue plasminogen activator, and raise plasminogen activator inhibitor-1 production (62). Nicoloff et al. showed that hypertensive children have lower serum cobalt concentrations than references (63). Glenn et al. revealed that changes in systolic blood pressure at baseline were associated with baseline lead concentration in blood and bone (64). Schober et al. demonstrated that blood lead levels as low as 5–9 μg/dL are associated with an increased risk of death from all causes, including cardiovascular disease, and cancer in a nationally representative sample of the U.S. population (65).

Nervous system

Several heavy metals, such as lead, mercury, cadmium, arsenic and aluminum, are known or suspected to have developmental neuro-toxicity. Neurodevelopmental deficits of children from e-waste recycling area have aroused great concern of public around the world. Children may have been exposed to hazardous e-waste components and by-products when living and playing in e-waste recycling environment, which pose significant health risks to children from fetal development to the end of adolescence (10,66,67). The mean scores of activity level, approach-withdrawal, and adaptability showed significant differences between Guiyu (exposed area) and Chengdian (reference area) children

according to one of our previous studies (68). In addition, associations have been found among BLLs, temperament alteration and the e-waste recycling activities in Guiyu (22). Neonates in Guiyu had significantly higher levels of umbilical cord blood and meconium lead and lower neonatal behavioral neurological assessment (NBNA) scores than those in the reference group. Moreover, NBNA scores were negatively correlated with BLLs in neonates of Guiyu. Though no correlation was found between umbilical cord blood lead and NBNA scores, a negative correlation was found between meconium lead and total NMNA, activitytone, and behavioural scores (69,70). Child blood levels of Pb, Cd, and Mn in Guiyu were correlated with certain behavioral abnormalities, such as conduct problems and antisocial behavior, and serum S100β were associated with blood heavy metals, and certain behavioral abnormalities (68).

The fact that maternal exposure to hazardous substances may affect the unborn child furthermore implies that exposure to e-waste may cause adverse health effects across generations. The mean BLLs of 1-to-6 years of age children living in Guiyu reaches 15 μg/dL, which is 50% higher than the neighboring reference site (~ 10 μg/dL) (18,21). BLLs $\geq$ 10 μg/dL in early childhood are detrimental to neurodevelopment, and recognized adverse effects include impaired cognitive function, behavioral disturbances, attention deficits, hyperactivity, and conduct problems. Preschool child blood Pb levels strongly predict neurologic deficits in children and young adults (71). There is a considerable amount of evidence showing that every 10- μg/dL increase of blood Pb concentration is associated with a deficit of 2–3 IQ points (72). Pb exposure in children has also been associated with increased risk of attention deficit hyperactivity disorder (73). A year ago, the U.S. Centers for Disease Control and Prevention (CDC) recommended the use of 5 ug/dL as a reference level that be used to trigger interventions, but recognized that no level of lead has been found to be safe (74). A recent study indicates that current background Cd exposure concentration in the U.S. is close 0.2 μg/L. However, in a Chinese birth cohort study, higher

Cd exposure in cord blood (> 0.6 µg/L) was associated with a 4-point full-scale IQ deficit at preschool age, after adjustment for cord blood Pb levels (75). The average blood Cd level (1.6 µg/L) in children from an e-waste recycling site in China was significantly higher than the reference site (1.0 µg/L). Reduced S100β, a Ca^{2+}-binding protein and potential biological indicator of heavy metal environmental pollution in specific neural lesions, was demonstrated in children with high blood Cd (76). Living in an e-waste recycling site substantially increases exposure of children to Cd, but the neurodevelopmental effects remain to be determined. Increased urinary 8-hydroxy-2´-deoxyguanosine, a biomarker for oxidative DNA lesions, was reported in children with high urinary Cr (77). E-waste recycling can result in high Cr exposure in fetuses, with one report of a mean cord blood Cr concentration of 99 µg/L, significantly higher than the mean cord blood Cr of the reference site (32 µg/L) (69,70). The reported Cr levels were very high compared with findings from a large U.K. study (serum ~ 0.5 µg/L) and Italian Cr workers (whole blood ~ 6.9 µg/L) (78-80).

Immune system

Heavy metal exposure has the potential immunotoxicity to humans and animals, especially in children. The interaction of heavy metals with the immune system, in an antigen-non-specific pattern, may lead to immunotoxicity which is the study of adverse effects on the immune system resulting from occupational or inadvertent exposure to heavy metals, drugs, chemicals and, in some instances, biologic materials. Heavy metals such lead, cadmium and aluminum inducing the alterations in immune system has been a matter of increasing scientific and public concern. As such, there have been marked efforts in basic research as well as incorporation and development of appropriate test methods to assess the potential immunotoxicity in experimental animals, wild life studies and humans. Significant effects of heavy metals on the immune function mainly include autoimmunity, oral tolerance, hypersensitivity and erythrocyte immune function, and expression of immune cells. Waterman et al. have shown that lead exposure results in the production of

autoantibodies against neural proteins, including myelin basic protein and glial fibrillary acidic protein, concluding that lead can aggravate neurological disease by increasing the immunogenicity of nervous system proteins (25). Fischbein et al. report that individuals with mildly elevated blood lead levels (>25 µg/dL) have detrimental effects on the host immune system, e.g. a reduction in absolute number and percentage of $CD3^+$ and $CD4^+$ cells in lead exposed individuals (81). Li et al. also find a decrease of $CD4^+$ T lymphocytes in children exposed to lead (82). Our recent study demonstrate that blood hepatitis B surface antibody levels were negatively associated with blood lead levels, and positively associated with age in children from an e-waste recycling area in China (20). These results suggest that exposure to environmental lead results in alterations in immune function of young children.

Reproductive system

Lead can cause infertility both in men and women at high doses (83-86). Lead is positively associated with increased rates of hypospermia, asthenospermia, and malformed sperm, as well as decreases in motility, penetrability, and number of sperm (82,87-89). Lead exposure can also affect chromatin stability in sperm nuclei (90,91). Lower doses of lead in women have been associated with increased rates of spontaneous abortion, reduced neonatal growth and neurological dysfunction in young children (87,92,93). Cadmium in the umbilical cord blood influenced Apgar 5-minute score, crown-heel length, birth weight and small-for-gestational age after adjustment for potential confounders in logistic regression models, and placental mercury levels significantly influenced head circumference, the Apgar 5-minute score and cord length (94). Studies by our team describe an increased median level of cord blood lead (CBPb) among neonates in Guiyu compared to a reference area, as well as lower Apgar scores and higher rates of adverse birth outcomes, such as stillbirth and low birth weight (95). A possible correlation was found between exposure to e-waste and changes in miRNA (small, non-coding RNAs involved in the regulation of gene expression during translation) expression profiles in spermatozoa. Out of

182 miRNAs identified from the e-waste exposed population, 109 were downregulated and 72 were upregulated compared to the reference population, indicating a potential association between ecological exposure to e-waste and sperm quality and count (96).

Skeletal system

Children are assigned to environmental pollution susceptible populations because of their unique physiological characteristics. Heavy metals, such as lead and cadmium, may interfere with normal growth and development of children. Long-term high cadmium exposure may cause skeletal damage, first reported from Japan, where the itai-itai (ouch-ouch) disease (a combination of osteomalacia and osteoporosis) was discovered in the 1950s. The exposure was caused by cadmium-contaminated water used for irrigation of local rice fields. A few studies outside Japan have reported similar findings. During recent years, new data have emerged also suggesting that relatively low cadmium exposure may give rise to skeletal damage, as evidenced by low bone mineral density (osteoporosis) and fractures (17,97-100). Yule et al find that children show slower growth with the higher blood lead levels (101). In a recent study by Yang et al., showed that BLLs were negatively associated with height and weight, however, positively associated with bone resorption biomarkers in the multiple linear regression analysis adjusted for age and sex among aged 3–8 children in Guiyu, which was regarded as an indication that e-waste exposure may have detrimental consequences on child physical development (17).

Urinary system

Inhalation, absorption or ingestion of heavy metal fumes or particles can be life threatening, and although acute pulmonary effects and deaths are uncommon, sporadic cases still occur. Heavy metal exposure can cause kidney damage such as an initial tubular dysfunction evidenced by an increased excretion of low molecular weight proteins [such as β_2-microglobulin and α_1-microglobulin (protein HC)] or enzymes [such as

N-acetyl-β-D-glucosaminidase (NAG)], which progresses to decreased glomerular filtration rate (GFR). It has been suggested that the tubular damage is reversible (102), but there is overwhelming evidence that the cadmium-induced tubular damage is indeed irreversible (103-105). In addition, cadmium can increase the risk of stone formation or nephrocalcinosis (14,106,107) and renal cancer (108,109). Several reports have shown that kidney damage and/or bone effects are likely to occur at lower kidney cadmium levels. European studies have shown signs of cadmium-induced kidney damage in the general population at urinary cadmium levels of 2–3 μg/g creatinines (27,110-112). Metallic mercury may cause reversible kidney damage, which is dependent on exposure levels of metallic mercury (14,113,114).

Conclusions

This brief review presents the adverse effects of a number of heavy metal pollutants from e-waste recycling area in children health. As shown, major injury of diverse organs can be observed. In addition, the genetic damage of heavy metals from e-waste recycling has aroused great interest of scientists and concern of public all over the world. The frequency of micronuclei in binucleated cells, measured by the cytokinesis-block micronucleus (CBMN) assay, is used as a marker for genotoxic (DNA) damage, especially at the chromosomal level (115). The comet assay (single cell gel electrophoresis) is used to detect damage in DNA in single cells, which is measured by tail length and rate. In addition, damage to DNA can be detected through chromosomal aberrations (fragments, monomers, translocations, satellites, and quadriradials) (116). Significantly higher micronuclei (median 4.0 ‰, range 2.0-7.0 ‰) in peripheral blood lymphocytes are found in residents from the e-waste recycling town of Guiyu when compared to a reference population with no involvement in e-waste recycling activities (median 1.0 ‰, range 0.0-2.0 ‰) (117). Another report demonstrates that significant differences in lymphocytic DNA damage in neonates are found in Guiyu compared to neighboring Chaonan. Guiyu neonates have greater DNA damage with significantly higher injury rates (33.20 %) and lengths of tails (4.49 ±1.92)

in the comet assay compared to neonates from Chaonan (10.70% and 2.09 ± 0.65, respectively). Additional significant correlations have been found between blood chromium levels and DNA damage in all study neonates.

E-waste is an emerging issue promoted by the rapidly increasing quantities of complex end-of-life electronic products. Informal e-waste recycling lead to large flows of toxic substances and poses health risks to exposed populations. The health impact of exposure to e-waste must become a priority of the international community. The specific impact of e-waste exposure on child health has not been adequately investigated. Although each of the assessed countries needs to develop expertise to tackle its potential e-waste management problems, most countries already have existing specific expertise that can be used and shared. Moreover, an international research agenda must be established by experts to increase the body of evidence and create strong scientific backing for combined international action. To optimize learning and maximize the efficiency of support for implementing improvements, a knowledge partnership in e-waste management is proposed in the form of an international WEEE Competence Centre. To develop possible new models for e-waste management to reduce e-waste exposure and its ensuing health effects, the international health community, academia, policy experts and nongovernmental organizations, in conjunction with national governments, should then cooperatively intervene and create policy solutions based on strong scientific evidence, which will benefit users, manufacturers, and recyclers in all countries.

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Notes

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CHAPTER 4

Decreased Lung Function with Alternation of Blood Parameters Linked to E-waste Lead and Cadmium Exposure in Preschool Children

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Abstract

Blood lead (Pb) and cadmium (Cd) has been associated with lung function in adults and smokers, but whether this also holds for children from electronic waste (e-waste) recycling area still unknown. We aimed to investigate associations between living area (e-waste exposed vs. reference areas), the levels of blood parameters (Pb, Cd, hemoglobin, and platelets) and the lung function levels (the forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV_1)). We recruited 206 preschool children from Guiyu (exposed area) and Haojiang and Xiashan (reference areas). Preschool children living in e-waste exposed areas have a risk of a 1.30 μg/dL increase in blood Pb and a 0.05 × 10^9/L increase in platelet account, while have a risk of a 3.31 g/L decrease in hemoglobin, 83 mL decrease in FVC and 73 mL decrease in FEV_1. Each unit of hemoglobin (1 g/L) decline was associated with 5 mL decrease in FVC and 4 mL decrease in FEV_1. We conclude that children living in e-waste exposed area have higher levels of Pb and platelets, and lower levels of hemoglobin and lung function. Hemoglobin may be a mediator when investigating the association between living area and lung function levels.

Keywords: Lead; Cadmium; Lung function; E-waste; Guiyu; Preschool children

Introduction

Electronic waste (e-waste), waste from electrical and electronic equipment or products after reaching the end of their useful life, has become the largest growing amount of waste in the world [1-4]. It was estimated that global e-waste generation was 41.8 million tonnes in 2014 and increase at the rate of about 16% annually [5-7]. E-waste cannot be considered or treated like any other waste because it contains blends of hazardous metals and plastics (organic pollutants), which are released into the local environment by roasting, burning, dismantling, acid leaching, inappropriate shredding, and dumping [8-10]. Heavy metals not only cannot be degraded into less hazardous end products but also can accumulate in environmental media such as air [11-13], dust [14-16], soil [16], water [17,18], and sediment [18,19], and in the human body through inhalation, ingestion, dermal absorption, and food chains [3,20]. As an example, lead (Pb) and cadmium (Cd) can enter the human body through respiration in the form of aerosol, dust, and steam, deposit into bronchioles and alveolar in the pulmonary airways, and concentrate in tissues and organs via the blood circulation. Environmental Pb and Cd exposure can be assessed by blood Pb and Cd levels [21]. Meanwhile, blood Pb and Cd levels are indicative of internal Pb and Cd exposure in Pb-and Cd-exposed individuals, respectively [22]. Heavy metal exposure may contribute to increased oxidative stress, disruption of barrier mechanisms, and inflammation and tissue destruction in the lungs [23]. Only a few studies have demonstrated that blood Pb and blood Cd were associated with lung function decline in adults, but whether this also holds for more susceptible children from electronic waste (e-waste) recycling area was neither explored nor know [24-26].

Guiyu is one of the largest e-waste destination and recycling areas in the world, and has a 30-year history of e-waste disposal and recycling with little or no regulation, and faces the highest levels of e-waste pollution in the world [27-29]. Previous studies reported concentrations of Pb and Cd in $PM_{2.5}$ [11-13], dust [14,16], soil [16], water [17,18], and sediment [18,19] were at a high level in Guiyu when compared to many other areas. Several cross-sectional studies demonstrated blood Pb and Cd levels

were serious in Guiyu for years [29-32]. One of our previous studies showed the level of lung function (forced vital capacity (FVC)) and the three kinds of heavy metals chromium, nickel and manganese in 144 primary school children in Guiyu and a reference area [33]. In this study, we concluded that there was no difference in FVC of children between the exposed and reference area. However, FVC was significantly lower in children living in Guiyu compared to the reference area in the youngest age group (8-9 years). It seems like that young children are particularly more sensitive and vulnerable population because of additional routes of exposure, high-risk behaviors, and their changing physiology compared to older children and adults [3]. Another recent research found that both the levels of lead and cadmium in $PM_{2.5}$ and in blood and the prevalence of respiratory symptoms cough, dyspnea, phlegm and wheeze in preschool children (3-to-8-years of age) were higher in Guiyu than the reference area [12]. In view of the lack of reports concerning association between blood Pb, blood Cd, and level of lung function in children from e-waste recycling areas, in the present study we recruited 206 preschool children (5-7 years of age) from Guiyu (the exposed area), and Haojiang and Xiashan (the reference areas) to extend our examination of heavy metals to lead and cadmium, and explore the association between blood Pb, blood Cd, living area and lung function in this age group.

In the present study, we measured the levels of blood parameters such as Pb, Cd, hemoglobin, platelet count, red blood cell distribution width (RDW), and performed spirometry to obtain FVC and forced expiratory volume in 1 second (FEV_1) in 206 preschool children aged 5-7 years from the exposed and reference areas. The purpose of this study was to investigate: (1) the levels of blood Pb, Cd, hemoglobin and platelet count and lung function in children from an e-waste exposed area and the two reference areas; (2) the association between the level of blood parameters and lung function parameters; (3) the association of living area with the levels of the blood parameters; (4) the association between living area and level of lung function and whether this association was mediated by the levels of the blood parameters.

Materials and methods

Study areas and population

The sampling site was located in Guiyu town, Shantou city, in the southeastern coast of Guangdong province in China. Haojiang (the first reference area) is located 31.6 km to the east of Guiyu; Xiashan (the second reference area) is located 5.8 km to the southeast of Guiyu (Figure 1). A total of 206 preschool children, 5-to-7-years old, were respectively recruited from three kindergartens from Guiyu (n=100), Xiashan (n=54), and Haojiang (n=52) during the period of November 2013 and December 2013. Participant received a general health questionnaire. All parents or guardians of the participants gave their written informed consent after receiving detailed explanations of the study and potential consequences prior to enrollment. This study protocol was approved by the Human Ethical Committee of Shantou University Medical College, China.

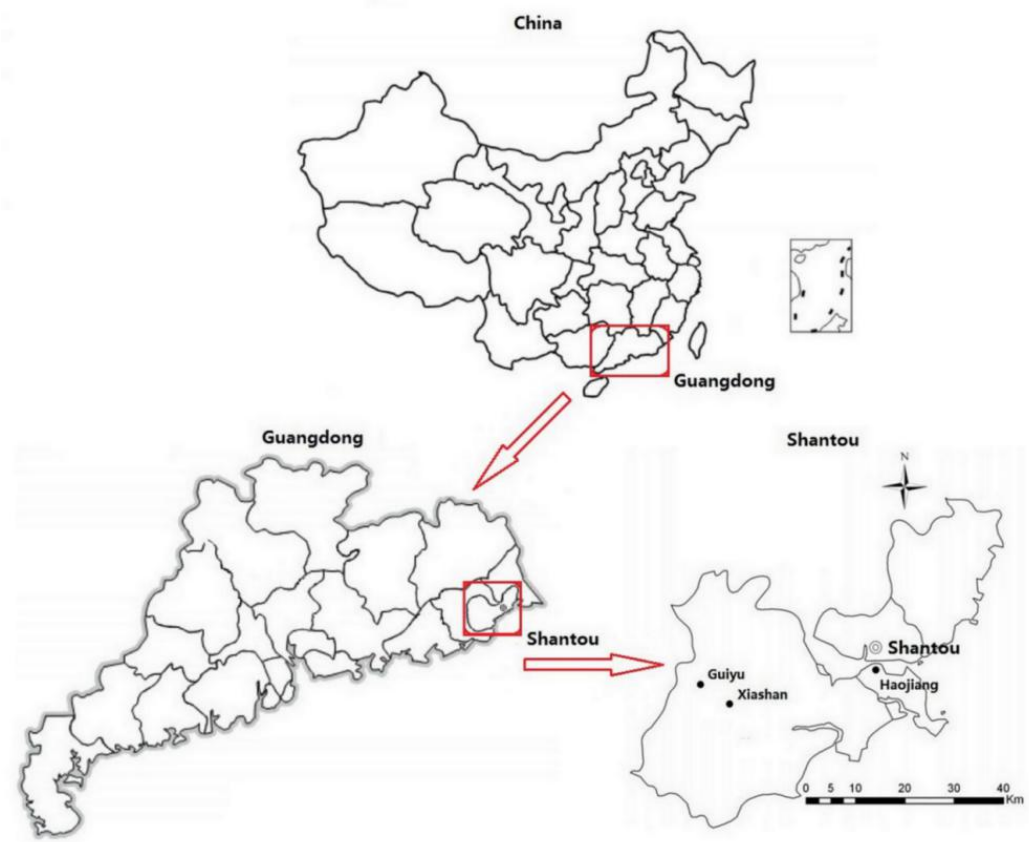

Figure 1. The location of the sampling sites (Guiyu, Haojiang and Xiashan).

Physical growth and development test

Physical growth and development parameters such as body height, body weight, and chest circumferences were measured at the time of blood sample collection. Height and weight were measured using a weight and height scale (TZ120; Yuyao Balance Instrument Factory, Yuyao, China). Children were required to remove their shoes, take off coat, and maintain a certain standing posture prior to being measured. All measurements were carried out by the trained physician [34].

Spirometry

Spirometry was conducted with a portable spirometer (Jiangsu Jintan Medical Instrument Factory, Model DF-II, Jintan, China), using standardized procedures following ATS-criteria [35]. Participants practiced the use of the mouthpiece with the spirometer until they felt comfortable under the guidance of the field physician. Results of three readings were recorded, and the highest reading of forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV_1) was used for analysis.

Questionnaire

We collected information on potential routes of exposure to e-waste, lead and cadmium, as well as general demographic and health parameters with a general questionnaire [12]. The basic information collected by the questionnaire have included age, gender, birth height, birth weight, parental smoking, residential history, dietary habits, parent's education and occupation, socioeconomic status, distance from home to a road, and whether home used as an e-waste recycling workshop. The parents or guardians of the children recruited in this study completed the questionnaire.

Blood sample analyses

Venipuncture was performed by nurses, and blood was stored at minus 20˚C until analysis. Hemoglobin, hematocrit, platelet counts, and thrombocytocrit were measured using an automatic hematology analyzer (Sysmex XT-1800i). Blood Pb and blood Cd determinations were

performed by graphite furnace atomic absorption spectrophotometry (Jena Zenit 650). The detailed procedure for measuring blood Pb and blood Cd was previously described in our previous studies [12,36].

Statistical methods

The association between living area (the exposed vs. reference areas) and levels of the blood parameters were analyzed by the basic linear regression model adjusted for age and gender. The associations between living area, the blood parameters, and the levels of lung function were tested by the basic linear regression models adjusted for age, gender, and height. Socioeconomic status, nutrition and other possible confounders were included in the each final model when they changed the estimate for blood parameters or living area by more than 10% or when the p-value of the confounder was lower than 0.05. Finally, linear regression models were performed with and without the blood parameters to investigate the influence of the blood parameters on the association between living area and lung function. P-values < 0.05 were considered statistically significant. All analyses were performed in SPSS version 22.0 (IBM Corporation, NJ, USA).

Results

General characteristics of participants in the exposed and reference areas

Parents of children had a lower educational level and family income, while had a higher prevalence of current smokers from the exposed area compared to the reference areas (Table 1). The general characteristics of the 206 preschool children are shown in Table 2. Average age of children from the three areas was comparable. Children had a lower birth weight and body height from the exposed area than the reference areas (Table 2).

Table 1. Demographic Characteristics of the Study Population (n=206).

Characteristic	Reference areas	Exposed area	Total	P-value
Subjects, n (%)	106 (51)	100 (49)	206 (100)	
Age (years)	5.39 (5.18-5.73)	5.49 (5.19-5.78)	206 (100)	0.881[a]
Gender (M/F), n (%)	63 (59)/43 (41)	52 (52)/48 (48)	206 (100)	0.283[b]
Parental smoking, n (%)	49 (46)	70 (70)	119 (58)	0.000[a]
Paternal education (n, (%))	105 (99)	95 (95)	200 (97)	0.000[a]
Illiteracy	2 (2)	0 (0)	2 (1)	
Primary school	4 (4)	6 (6)	10 (5)	
Middle school	24 (23)	60 (64)	84 (42)	
Secondary school	9 (9)	13 (14)	22 (11)	
High school	22 (21)	7 (7)	29 (15)	
College/university	44 (41)	9 (9)	53 (26)	
Maternal education (n, (%))	105 (99)	95 (95)	200 (97)	0.000[a]
Illiteracy	2 (2)	1 (1)	3 (2)	
Primary school	11 (10)	11 (12)	22 (11)	
Middle school	22 (21)	63 (66)	85 (43)	
Secondary school	20 (19)	12 (13)	32 (16)	
High school	19 (18)	4 (4)	23 (11)	
College/university	31 (30)	4 (4)	35 (17)	
Family income level (n, (%))	103 (97)	87 (87)	190 (92)	0.001[a]
< 1500 ¥ per month	3 (4)	5 (5)	8 (4.7)	
1500-3000 ¥ per month	14 (13)	24 (24)	38 (19.7)	
3000-4500 ¥ per month	19 (20)	18 (18)	37 (20.2)	
4500-6000 ¥ per month	18 (17)	18 (18)	36 (18.7)	
> 6000 ¥ per month	49 (46)	22 (22)	71 (37.7)	

Reference areas: Haojiang and Xiashan; Exposed area: Guiyu; a: Mann-Whitney U test; b: Pearson χ^2 test.

The levels of blood Pb and blood Cd between the exposed and reference areas

The average concentrations of blood Pb and the ratio of blood Pb > 5 μg/dL in children was higher from the exposed area than the reference areas, while the level of blood cadmium showed no significant difference in children between the exposed and reference areas (Table 2).

The levels of other blood parameters between the exposed and reference areas

The level of hemoglobin in children's blood was lower in the exposed area than the reference areas. The level of platelet and RDW in children's blood was higher in the exposed area than the reference areas (Table 2). The ratio of thrombocytosis (platelet count > 400×10^9/L) for children was higher in the exposed area than the reference areas.

The levels of lung function between the exposed and reference areas

The lung function parameters (FVC and FEV_1) were lower in children from the exposed area than the reference areas, whereas FVC/FEV_1 showed no significant difference in children between the exposed and reference areas (Table 2).

Associations between living area and the levels of blood parameters

Table 3 shows that living in Guiyu was associated with a higher level of blood Pb and platelet count and a lower level of hemoglobin in the basic model and in the respective full models (Table 3).

Table 2. Levels of physical parameters, blood parameters, and lung function parameters in preschool children from Guiyu, Haojiang and Xiashan (n=206).

Characteristic	Reference areas	Exposed area	P-value
Subjects, n (%)	106 (51)	100 (49)	
Physical parameters			
Birth Height (cm)	51.87 ± 5.37	50.21 ± 3.96	0.071[a]
Birth Weight (kg)	3.25 ± 0.58	3.07 ± 0.49	0.020[a]
Body height (cm)	112.56 ± 4.70	111.03 ± 5.95	0.045[a]
Body weight (kg)	19.76 ± 2.46	19.30 ± 2.84	0.412[a]
BMI (kg/m^2)	15.12 (14.35 ~ 16.24)	15.43 (14.86 ~16.33)	0.128[c]
Blood parameters			
Pb (μg/dL)	3.57 (2.68 ~ 4.86)	5.53 (3.92 ~ 7.04)	0.000[b]
Pb > 5μg/dL, n (%)	26 (24.5)	58 (58.0)	0.000[c]
Cd (μg/L)	0.57 (0.42 ~ 0.79)	0.58 (0.44 ~ 0.79)	0.717[b]
Hemoglobin (g/L)	131.49 ± 10.02	128.17 ± 8.28	0.013[a]
Hematocrit (%)	39.99 ± 2.73	39.27 ± 2.33	0.048[a]
Blood platelet (10^9/L)	278.40 ± 77.86	338.03 ± 82.11	0.000[a]
Thrombocytosis, n (%)	5 (4.7)	20 (20.0)	0.001[c]
Thrombocytocrit	0.28 (0.24 ~ 0.32)	0.33 (0.29 ~ 0.38)	0.000[c]
RDW (%)	12.40 (12.10 ~12.80)	12.79 (12.40 ~13.10)	0.002[c]
Lung function levels			
FVC (L)	1.33 ± 0.25	1.23 ± 0.24	0.004[a]
FEV_1 (L)	1.24 ± 0.23	1.16 ± 0.21	0.005[a]
FEV_1 / FVC	0.96 (0.92 ~ 0.98)	0.95 (0.91 ~ 0.97)	0.206[c]

Reference areas: Haojiang and Xiashan; Exposed area: Guiyu; a: T-test; b: T-test based on Ln-transformation; c: Mann-Whitney U test; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 second; FEV_1/FVC: the ratio of forced expiratory volume in 1 second to forced vital capacity; Pb: lead; Cd: cadmium. Thrombocytosis: platelet count > 400×10^9/L. RDW: red blood cell distribution width. For continuous variables, average range is presented as mean values ± SD (normal distribution data) or median values with interquartile (non-normal distribution data).

96 **Table 3.** The association between living areas and blood parameters (n=206).

Living area	lnBPb B (95% CI)	lnBCd B (95% CI)	Hemoglobin B (95% CI)	Platelet count B (95% CI)
Univariate Model	0.47 (0.29 ~ 0.53)***	0.03 (-0.09 ~ 0.15)	-3.32 (-5.93 ~ - 0.71)*	0.06 (0.04 ~ 0.08)***
Basic Model	0.41 (0.29 ~ 0.53)**	0.03 (-0.10 ~ 0.15)	-3.44 (-6.06 ~ - 0.82)**	0.06 (0.04 ~ 0.09)***
Full Model	0.26 (0.10~ 0.41)**	-0.09 (-0.25 ~ 0.08)	-3.58 (-6.76 ~ -0.40)*	0.05 (0.01 ~ 0.08)**

Abbreviations, lnBPb: ln-transformation of blood lead; lnBCd: ln-transformation of blood cadmium; Platelet count unit: $(10^9/L)/(1000)$; CI: Confidence intervals; Living area: Guiyu vs. Haojiang and Xiashan; Basic Model: Adjusted for age and gender; Full Model: Adjusted for age, gender, red blood cell distribution width (RDW), parental smoking, family incomes, parental education levels, whether parents participated in e-waste recycling work, whether home worked as a workshop or close to an e-waste recycling site, and frequency of eating preserved eggs. ***: $p \leq 0.001$; **: $p \leq 0.01$; *: $p \leq 0.05$.

Associations between the levels of the blood parameters and lung function

Blood hemoglobin was positively associated with FVC and FEV_1 both in the univariate model, basic models and in the full models (Table 4). No significant association was found between blood Pb, Cd, platelet count and levels of FVC and FEV_1 (Table 4).

Associations between living area and the levels of lung function

Living in Guiyu was associated with lower lung function levels both in the univariate model, basic models and the full multivariate linear regression models (Table 5). Blood Pb, hemoglobin and platelet count were included in the each final model to assess whether these blood parameters mediated the association between living area and lung function levels. Analysis results showed that they changed the estimate for living area by more than 10% or the p-value of living area was changed from lower than 0.05 to higher than 0.05. In other words, blood Pb, hemoglobin and platelet mediated the associations between living area and the levels of lung function (Table 5).

Table 4. The association between blood parameters and lung function (n=206).

Blood parameters	FVC B (95% CI)	FEV_1 B (95% CI)
lnBPb		
Univariate Model	-0.047 (-0.117 ~ 0.024)	-0.052 (-0.116 ~ 0.011)
Basic Model	-0.042 (-0.107 ~ 0.023)	-0.049 (-0.109 ~ 0.011)
Full Model	-0.023 (-0.099 ~ 0.054)	-0.029 (-0.098 ~ 0.041)
lnBCd		
Univariate Model	-0.041 (-0.119 ~ 0.036)	-0.031 (-0.101 ~ 0.039)
Basic Model	-0.003 (-0.074 ~ 0.067)	-0.001 (-0.065 ~ 0.064)
Full Model	-0.018 (-0.093 ~ 0.057)	-0.016 (-0.084 ~ 0.053)
Hemoglobin		
Univariate Model	0.004 (0.000 ~ 0.008)*	0.003 (0.000 ~ 0.007)*
Basic Model	0.004 (0.001 ~ 0.007)*	0.003 (0.000 ~ 0.006)*
Full Model	0.005 (0.001 ~ 0.009)*	0.004 (0.001 ~ 0.008)*
Platelet count		
Univariate Model	0.088(-0.298 ~ 0.473)	0.169 (-0.178 ~ 0.515)
Basic Model	0.154 (-0.194 ~ 0.503)	0.226 (-0.094 ~ 0.546)*
Full Model	0.296 (-0.093 ~ 0.684)	0.337 (0.015 ~ 0.688)#

Abbreviations, lnBPb: ln-transformation of blood lead; lnBCd: ln-transformation of blood cadmium; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 second; CI: Confidence intervals; Basic Models : Adjusted for age, gender, height and living area; Full Model: Adjusted for age, gender, height, living area, red blood cell distribution width (RDW), parental smoking, family incomes, parental education levels, whether parents participated in e-waste recycling work, and whether home worked as a workshop or close to an e-waste recycling site. **: $p \leq 0.01$; *: $p \leq 0.05$; #: $0.05 < p \leq 0.10$.

Table 5. The association between living areas and lung function through blood parameters (n=206).

Living area	FVC B (95% CI)	FEV_1 B (95% CI)
Univariate Model	-0.098 (-0.165 ~ -0.031)**	-0.087 (-0.147 ~ -0.026)**
Basic Model	-0.064 (-0.125 ~ -0.002)*	-0.059 (-0.116 ~ -0.002)*
Full Model	-0.083 (-0.159 ~ -0.006)*	-0.073 (-0.143 ~ -0.004)*
Full Model+lnBPb	-0.076 (-0.156 ~ 0.005)#	-0.064 (-0.137 ~ 0.009)#
Full Model+lnBCd	-0.084 (-0.161 ~ 0.007)*	-0.074 (-0.144 ~ 0.005)*
Full Model+Hemoglobin	-0.066 (-0.143 ~ 0.011)#	-0.058 (-0.128 ~ 0.011)#
Full Model+Platelet count	-0.094 (-0.172 ~ 0.016)*	-0.086 (-0.157 ~ 0.016)*

Abbreviations, lnBPb: ln-transformation of blood lead; lnBCd: ln-transformation of blood cadmium; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 second; CI: Confidence intervals; Living area: Guiyu vs. Haojiang and Xiashan; Basic Model: Adjusted for age, gender, and height; Full Model: Adjusted for age, gender, height, red blood cell distribution width (RDW), parental smoking, family incomes, parental education levels, whether parents participated in e-waste recycling work, and whether home worked as a workshop or close to an e-waste recycling site. **: $p \leq 0.01$; *: $p \leq 0.05$; #: $0.05 < p \leq 0.10$.

Discussion

This study investigated the levels of blood parameters (Pb, Cd, hemoglobin and platelet) and lung function (FVC and FEV_1), evaluated the associations between living area and the blood parameters, the associations between the blood parameters and levels of lung function, the associations between living area and levels of lung function, and assessed whether the blood parameters mediated the association between living area and levels of lung function in the e-waste exposed and reference areas. Firstly, we found that blood Pb and platelet were higher from the exposed area than the reference areas. While hemoglobin in children's blood was lower from the exposed area than the reference areas. Second, living in Guiyu was associated with higher levels of Pb and platelet, and lower levels of hemoglobin and lung function. Moreover, lower blood hemoglobin level was associated with

lower lung function level. Furthermore, both blood Pb, hemoglobin and platelets mediated the association between living area and lung function levels.

Although blood Pb in children from the e-waste exposed area was as low as 5.53 μg/dL in the current study, was still higher than the reference areas and the new CDC guidelines (5 μg/dL) [37]. The CDC guidelines of blood lead have been reduced from 10 μg/dL to 5 μg/dL, which indicates no level of lead exposure appears to be 'safe' [37]. The average concentration of blood Pb in children from Guiyu now was lower than blood Pb in 2004 (15.30 μg/dL), 2006 (13.17 μg/dL), 2009 (7.30 μg/dL), 2011 (6.76 μg/dL), and 2012 (6.24 μg/dL) according to our previous cross-sectional studies, which may due to the strict policies promulgated and enforced by the local government in the last decade [12,29-31]. However, there were still 58.0% of the children in Guiyu have a blood Pb > 5 μg/dL in the current study, which indicated that lead derived from e-waste in Guiyu is still very serious [37]. Similar to blood Pb, the average concentration of children's blood Cd (0.58 μg/L) from Guiyu in this study was lower than blood Cd in 2006 (1.58 μg/L) and blood Cd in 2009 (0.69 μg/L) [30,31], and firstly demonstrated no difference with blood Cd from the reference areas in this study, which may because that blood Cd in children from Guiyu keep stable in the past four years, and partly ascribe to the increase of blood Cd in the reference areas (i.e. Median of blood Cd from Haojiang was 0.50 μg/L in 2012, and 0.57 μg/L in 2013) [12]. Over 99% of the children have blood Cd > 0.2 μg/L (the latest suggest toxicity threshold value of blood Cd), which was still higher than the blood Cd of children from America (0.23 μg/L), Africa (0.21 μg/L), and Europe (0.15 μg/L) [38,39]. It is necessary to continue to monitor cadmium exposure in these research areas.

It has been found that heavy metals such as Pb and Cd can disturb the synthesis of hemoglobin through impairing heme synthesis and the production of erythropoietin [40-43]. Heme is a cofactor, component, and

the main stabilizing structural molecule of hemoglobin. Erythropoietin is a glycoprotein hormone that regulates erythropoiesis or red blood cell production. Associations between heavy metals and hemoglobin have been investigated by a few studies in adults, but the results are inconsistent and inconclusive [44-46]. In the current study, we investigated the association between blood heavy metals and hemoglobin in preschool children from an e-waste exposed area and two reference areas. The results showed that there was just a weak correlation between blood Cd and hemoglobin (Table S1). The complex toxicokinetics and co-exposure of heavy metals, and its nonlinear relationship with health effects may explain the part of the reasons [47]. In addition, e-waste, a kind of composite exposure source, not only contains heavy metals but also releases organic pollutants such as polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) into the environment, which inevitably affects the blood hemoglobin level. For example, Leijs et al showed that the total PBDEs were positively associated with the hemoglobin concentration in a cohort of mother-baby pairs [48]. Xu et al demonstrated that both the mean level of total PBDEs and PCBs was positively correlated with levels of hemoglobin and platelets in the individuals from an e-waste dismantling area in China [49].

Hemoglobin, the primary transporter of oxygen, is essential for oxygen transport for carrying oxygen from the respiratory organs (i.e. lungs) to the rest of the body (i.e. the tissues) [50]. Hassel et al. showed that there was a positive association between lung function and peak oxygen uptake in elderly [51]. Guo et al. found low level of blood hemoglobin was related to gas exchange decline and the quantity of oxygen uptake in COPD patients [52]. In addition, hemoglobin has a critical role in vascular remodeling and pulmonary hypertension [53-55]. Low hemoglobin levels were associated with higher risk for adverse cardiovascular outcomes, and reduced exercise capacity and health related quality of life (HRQL) [56,57]. Therefore, we proposed that low level of blood hemoglobin may contribute to lung function decline. In this study, we found each unit of

hemoglobin (1 g/L) decline was associated with 5 mL decrease in FVC and 4 mL decrease in FEV_1 in preschool children from the e-waste recycling and two reference areas. Blood hemoglobin levels may be able to be used as a predictor for lung function level.

Although we found no association between platelet count and lung function levels in the final linear regression model, we found an association between platelet count and lung function levels in the basic linear regression model. In addition, blood platelet count was higher in Guiyu than the reference areas. Platelets are inflammatory cells with an important role in antimicrobial host defenses [58]. Elevated platelet count and thrombocytosis are often considered a marker of normal inflammatory reaction of infections, and has been associated with increased mortality in hospitalized patients with community-acquired pneumonia [59]. The elevated platelets and thrombocytosis in children' blood from Guiyu may indicate that children from Guiyu were at higher risk of infections and undergone higher levels of inflammatory reactions compared to the reference areas. Furthermore, the prevalence of thrombocytosis (platelet count > 400×10^9/L) in children was higher in Guiyu compared to the reference areas. Several studies reported that thrombocytosis is positively associated with acute and chronic inflammation in vessels, which may indicate that the prevalence of inflammation in vessels may be higher in children exposed to e-waste [60,61].

Heavy metals such as Pb and Cd can generate reactive oxygen species (ROS) via losing electrons or activate generation of ROS when heavy metals phagocytozed by alveolar macrophages [62]. ROS could cause damage to pulmonary capillary endothelial cells, alveolar epithelial cells and basilar membrane cells. It also leads to alveolar collapse, atelectasis and alveolar ventilation dysfunction [63]. Several studies demonstrated that heavy metals such as Pb and Cd were associated with lung function level in adults. For instance, Pak et al demonstrated that the blood lead

level was associated with the decline of FVC in 263 adults aged over 30 [24]. Oh et al showed that blood cadmium level was associated with deteriorated lung function in males aged ≥ 40 years [26]. Leem et al reported that both blood lead and cadmium were associated with decreased lung function in subjects aged ≥ 20 years [64]. An inverse dose-response association was observed between urinary lead and FEV_1/FVC ratio [65]. Lead and manganese in atmospheric particulate matter (PM) were associated with a decrement of peak expiratory flow rate of schoolchildren [66]. We recruited 206 preschool children to confirm whether the association between blood Pb and Cd and lung function is also applicable to children, and the result showed that there was no significant association between blood Pb and Cd and lung function levels. This may be mainly due to that previous studies were based on the adult population such as occupational workers and smokers, which is different from general children population [3]. Children are not only considered to be more susceptible to hazardous environmental pollutants compared to adults but also in the stage of rapid growth and development of the lung. In addition, the complex biochemical toxicity of Pb may also contribute to the no association between blood heavy metals and lung function levels in preschool children in this study.

Most of the blood and lung function parameters showed a significant difference in children between Guiyu and the reference areas. Therefore living area may contribute to the alteration of blood and lung function parameters in children. We make a further investigation to assess whether living area was associated with the levels of blood parameters and lung function in children. It is known that both socioeconomic status [67] and nutrition [68] are important potential confounders for lung function, which cannot be ignored when studying lung function. Family income levels and parental education level were used as indicators for socioeconomic status in this study. Meanwhile, red blood cell distribution width (RDW) has been considered as a biomarker for nutritional status of vitamin B12, folic acid, and iron because that an increased RDW is associated with nutritional deficiency [69]. After adjusted for family income

levels, parental education level and RDW and other confounders in the multiple linear regression models, results showed that living in Guiyu was still associated with lower levels of hemoglobin and lung function, and higher levels of blood Pb and platelets of children. An interesting question comes out that whether blood Pb, hemoglobin and platelet act as a mediator mediated the association between living in Guiyu and lung function levels. The results indicated that these blood parameters can contribute to the association between living area and lung function levels.

Our study has some limitations. First, we recruited children in 5-7 years of age from Guiyu. Haojiang and Xiashan, and an even earlier age of preschool children were excluded to ensure the accuracy and stability of lung function measurements resulting in the small sample sizes. A larger population and more detailed individual measurements would be necessary to draw more accurate and powerful conclusions in future studies. Second, the cross-sectional nature of this study prevents us from drawing any conclusions about a cause-effect relation. Finally, we did not investigate the effects of the other heavy metals (chromium, nickel, mercury, and arsenic) and organic pollutants (polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzodioxins and dibenzofurans (PCDD/PCDFs), or polybrominated diphenyl ethers (PBDEs)) derived from e-waste on lung function levels and on the association between living area and lung function level due to insufficient blood quantities. However, to our knowledge, this is the first study to investigate the association between living area and lung function levels, and assess whether blood Pb, Cd, hemoglobin and platelet mediate the association between living area and lung function levels in preschool children from an e-waste exposed area.

In conclusion, the current results show that children from Guiyu have accelerated levels of Pb and platelets, and had lower levels of hemoglobin and lung function. In addition, a lower level of hemoglobin is associated with a lower level of lung function in this study. Due to the strict

local policy in Guiyu, the concentration of blood Pb in children is lower now compared to our previous studies reported in 2004, in 2006, in 2009, in 2011, and in 2012. The concentration of blood Cd in children from Guiyu is lower now compared to those reported in 2006, and in 2009, but showed no difference with blood Cd in 2012. These findings, along with evidence from previous studies, suggest that Pb and Cd poisoning continues to be a major public health problem for children in Guiyu. Broader consensus of the harm of Pb and Cd, proactive regulation and restriction of e-waste production and effective protection of individuals from e-waste are essential steps to steadily reduce blood Pb to the level under CDC guidelines, and let blood Cd under suggestive level. In addition, children who living in Guiyu is more likely to suffer from low lung function level. It is also imperative to recruit more children through their childhood to confirm whether pollutants such as heavy metals and plastics from e-waste have a long-term adverse effect on lung function and whether the early-life Pb and Cd exposure are associated with other health outcomes later in childhood.

Conflict of interest

All authors have read and approved this version of the article, and throughout the study, care has been taken to ensure the integrity of the work. This article or one with similar content has not been previously published or submitted to any other journal. All authors declare that they have no conflicts of interest. The study was approved by the Human Ethics Committee of Shantou University Medical College.

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Table S1. Spearman correlation analyses among the blood parameters and lung function levels in preschool children.

	BPb	BCd	Hemoglobin	Hematocrit	Platelets	Thrombocytocrit	FVC	FEV_1
BPb	1.000							
BCd	-0.003	1.000						
Hemoglobin	-0.059	0.203**	1.000					
Hematocrit	-0.078	0.152*	0.942**	1.000				
Platelets	0.083	0.024	-0.027	0.042	1.000			
Thrombocytocrit	0.140	-0.002	-0.027	0.041	0.911**	1.000		
FVC	-0.074	-0.087	0.145*	0.149*	0.001	-0.080	1.000	
FEV_1	-0.100	-0.099	0.128*	0.139*	0.047	-0.045	0.963**	1.000

BPb: blood lead; BCd: blood cadmium; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 second; **: $p \leq 0.01$; *: $p \leq 0.05$.

CHAPTER 5

Genome-wide association study to identify susceptibility loci for respiratory symptoms

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Abstract

Background: Respiratory symptoms are associated with accelerated lung function decline, and increased hospitalization and mortality rates in the general population. Although several environmental risk factors for respiratory symptoms are known, knowledge on genetic risk factors is lacking. We aim to identify genetic variants associated with respiratory symptoms by genome-wide association (GWA) analyses.

Methods: We conducted the first GWA study on cough, dyspnea and phlegm among 7,976 participants in the LifeLines I cohort and used the LifeLines II cohort (n=5,260) and the Vlagtwedde-Vlaardingen cohort (n=1,529) for replication.

Results: Rs16918212, located in the alpha-2-macroglobulin pseudogene 1 (*A2MP1*), was associated with cough in both the identification (odds ratio (OR) = 0.72, $p = 5.41 \times 10^{-5}$) and the meta-analyzed replication cohorts (OR = 0.83, p = 0.033). No significant associations with dyspnea and phlegm were found.

Conclusions: We did not find a convincing association between genetic markers and respiratory symptoms. Since, environmental exposures are important risk factors for respiratory symptoms; the next step is to perform a genome-wide interaction (GWI) study to identify genetic susceptibility loci for respiratory symptoms in interaction with known harmful environmental exposures.

Keywords: GWAS, genetic, respiratory symptoms, general population, cohorts

Background

The presence of respiratory symptoms, such as chronic cough, dyspnea and phlegm, is associated with lower lung function (1-2) and with mortality due to several causes of death (3-5). Respiratory symptoms have been regarded as important markers of accelerated lung function decline (6,7) and development of asthma (8).

It is known that cigarette smoking (9), allergy (10,11), air pollution (12,13) and occupational exposures (14-15) are risk factors for respiratory symptoms. However, not all exposed subjects develop respiratory symptoms, which suggest that a genetic component may be involved in the development of respiratory symptoms. Previous studies reported associations between respiratory symptoms and specific genetic loci using candidate gene studies (16,17). To date, only one genome-wide association (GWA) study has investigated genetic susceptibility of one phenotype (Chronic mucus hyper-secretion) of respiratory symptoms (18). Genetic susceptibility to develop respiratory symptoms such as cough, dyspnea, and phlegm has not been studied up till now using GWA methods.

In the current study, we conducted several GWA analyses, i.e. on cough, dyspnea and phlegm, in 7,976 Caucasians of Dutch descent from the large population-based LifeLines I cohort study to identify common genetic variants associated with respiratory symptoms. We used the LifeLines II cohort and the Vlagtwedde-Vlaardingen cohort to replicate our initial findings.

Methods

Identification cohort

Genotyped individuals from the first data release of the LifeLines cohort study (2006-2011, LifeLines I) with full data on all covariates were included (n = 7,976). The LifeLines cohort study is a prospective population-based cohort studying health and health-related behavior of subjects from the three Northern provinces of the Netherlands (19,20).

Replication cohorts

We included 5,260 subjects from the second data release from the LifeLines cohort study (2006-2011, LifeLines II) and 1,529 subjects from the last survey (1989/1990) from the Vlagtwedde-Vlaardingen cohort (21,22), a prospective general population based cohort including Caucasians of Dutch descent, to replicate our initial findings.

Ethics, consent and permissions

Participants provided written informed consent. The study was approved by the Medical Ethics Committee of the University Medical Center Groningen, Groningen, The Netherlands (ref. METc 2007/152).

Genotyping and quality control

Genotyping was performed in the identification and replication cohorts using IlluminaCytoSNP-12 arrays. Samples with call-rates of less than 95% were excluded. SNPs that fulfilled the quality control criteria were included: genotype call-rate ≥ 95% (on average a call-rate of >99% is achieved), minor allele frequency (MAF) ≥ 1%, and Hardy-Weinberg equilibrium (HWE) cut-off p-value ≥ 10^{-4}. Non-Caucasian samples and first-degree relatives were excluded from the LifeLines I and LifeLines II. Vlagtwedde-Vlaardingen did not include non-Caucasian samples or

first-degree relatives. A total of 227,981 genotyped SNPs were included in the identification analysis.

Respiratory symptoms

Cough, dyspnea, and phlegm were defined by standardized questionnaires. Cough was defined as at least one positive answer to the questions: “do you usually cough first thing in the morning in the winter?” or “do you usually cough during the day, or at night, in winter?”. Dyspnea was defined as a positive answer to the question: “are you troubled by shortness of breath when hurrying on level ground or walking up a slight hill or stairs at normal pace?”. Phlegm was defined as at least one positive answer to the questions: “do you usually bring up any phlegm from your chest first thing in the morning in winter?” or “do you usually bring up any phlegm from your chest during the day, or at night, in winter?”.

Statistical analysis

The data are presented as median (min-max) for continuous variables and as frequencies (percentages) for categorical variables. The GWA analyses on the presence of the respiratory symptoms cough, dyspnea, and phlegm were performed using PLINK version 1.07 (23). We used an additive genetic model adjusted for age, sex, and current smoking. SNPs with a p-value < 10^{-4} in the identification analysis were taken forward for replication. Replication analysis was performed by analyzing the two replication cohorts separately using logistic regression model in PLINK version 1.07 (23) and subsequently meta-analyzing effect estimates from both cohorts. Significant replication was defined as a fixed effect meta-analysis p-value < 0.05 and an effect estimate in the same direction as in the identification GWA study. SNP annotation was performed using HaploReg version 4 (Broad Institute).

Results

Demographic characteristics and the prevalence of respiratory symptoms in the study cohorts are summarized in Table 1. In the identification cohort LifeLines I, the median age of subjects was 48 years old, 43% was male, and 24% was a current smokers. The replication cohorts were comparable with the identification cohort with respect to demographic characteristics. The prevalence of respiratory symptoms in the LifeLines cohorts and Vlagtwedde-Vlaardingen cohort varied from 7% to 25%.

A total of 17 SNPs, 19 SNPs and 14 SNPs were identified for cough (Table 2), dyspnea (Table 3) and phlegm (Table 4) in the identification analyses in LifeLines I, respectively, and taken forward for replication in LifeLines II and Vlagtwedde-Vlaardingen. Rs16918212 (OR = 0.72, p = 5.41×10^{-5} in identification; OR = 0.83, p = 0.033 in replication), located on *A2MP1*, was significantly associated with cough in the replication cohorts with the same direction of effect as in the identification cohort (Table 2). The replication analyses on dyspnea and phlegm showed no significant replication (Table 3 and Table 4).

Table 1. Characteristics of the subjects included in the identification (LifeLines I) and replication (LifeLines II and Vlagtwedde-Vlaardingen) cohorts.

	Identification LifeLines I	Replication	
		LifeLines II	Vlagtwedde-Vlaardingen
N	7,976	5,260	1,529
Male, n (%)	3,420 (43)	2,112 (40)	714 (46)
Age (yrs), median (min-max)	48 (20-89)	48 (18-90)	51 (35-65)
Current smokers, n (%)	1,904 (24)	1,051 (20)	548 (36)
Smoking status, n (%)			
Ever	4,737 (59)	3,050 (58)	1,054 (69)
Never	3,239 (41)	2,209 (42)	475 (31)
Respiratory symptoms, n (%)			
Cough	675 (9)	409 (8)	175 (12)
Dyspnea	547 (7)	358 (7)	147 (10)
Phlegm	1,702 (21)	1,068 (20)	337 (25)

Table 2. Top strongest 17 SNPs ($P < 1.0\times10^{-4}$) be associated with cough in the GWA study.

SNPs	Chr	Gene	A1	MAF	Identification LifeLines I		Replication				Meta-analysis Replication	
							LifeLines II		Vlagtwedde-Vlaardingen			
					OR	P	OR	P	OR	P	OR	P
rs11813494	10	638kb 3' of SFTA1P	G	0.24	1.279	1.12E-06	1.110	0.114	0.776	0.096	1.049	0.427
rs840952	2	60kb 5' of SPRED2	G	0.10	1.212	1.09E-05	1.168	0.006	0.814	0.169	1.035	0.401
rs7633390	3	27kb 5' of ZNF717	T	0.18	1.237	2.24E-05	1.037	0.581	0.865	0.305	1.005	0.938
rs4977230	9	SLC24A2	T	0.71	0.817	3.35E-05	1.020	0.744	0.986	0.910	1.013	0.806
rs4780334	16	CIITA	G	0.69	1.209	3.78E-05	0.985	0.802	0.875	0.295	0.964	0.500
rs844033	15	126kb 5' of NDN	C	0.91	0.702	3.84E-05	1.163	0.115	1.213	0.342	1.172	0.068
rs422564	5	3.9kb 3' of TBCA	T	0.21	1.201	3.97E-05	1.035	0.549	1.196	0.123	1.065	0.222
rs12153520	5	77kb 3' of HINT1	A	0.16	1.259	4.48E-05	1.012	0.875	1.020	0.904	1.013	0.844
rs893966	1	EPHB2	T	0.50	1.248	4.72E-05	0.992	0.909	0.909	0.532	0.975	0.707
rs6775462	3	ROBO2	C	0.50	0.739	4.81E-05	0.952	0.588	1.124	0.497	0.987	0.869
rs4766584	12	ACACB	G	0.45	0.821	5.15E-05	0.929	0.234	0.994	0.964	0.941	0.274
rs16918212	12	A2MP1	A	0.20	0.717	5.41E-05	0.882	0.196	0.613	0.021	0.828	0.033
rs2623166	8	GEM	T	0.13	1.293	7.68E-05	0.994	0.948	0.635	0.029	0.928	0.360
rs17729233	18	DSG4	G	0.44	1.257	7.89E-05	1.034	0.668	0.997	0.982	1.026	0.714
rs2850106	21	HLCS	G	0.12	0.839	8.29E-05	1.050	0.384	0.998	0.989	1.040	0.434
rs10758982	9	PTPRD	G	0.34	1.211	8.36E-05	0.967	0.602	0.827	0.163	0.939	0.284
rs2845804	21	HLCS	C	0.18	0.841	9.93E-05	0.976	0.663	1.090	0.461	0.996	0.941

124 Table 3. Top strongest 19 SNPs ($P < 1.0\times10^{-4}$) be associated with dyspnea in the GWA study.

SNPs	Chr	Gene	A1	MAF	Identification LifeLines I		Replication				Meta-analysis Replication	
							LifeLines II		Vlagtwedde-Vlaardingen			
					OR	P	OR	P	OR	P	OR	P
rs10754237	1	LHX9	T	0.03	1.883	1.80E-06	0.921	0.642	1.523	0.057	1.120	0.412
rs658121	18	LAMA1	T	0.14	1.349	2.70E-06	1.039	0.638	0.776	0.070	0.965	0.614
rs6428425	1	8.1kb 3' of LHX9	G	0.97	1.864	2.90E-06	0.912	0.602	1.528	0.056	1.067	0.395
rs13388308	2	ATF2	T	0.19	0.751	4.59E-06	0.997	0.961	0.939	0.581	0.980	0.739
rs7236872	18	208kb 3' of CBLN2	A	0.53	1.237	7.79E-06	1.033	0.583	0.956	0.626	1.010	0.834
rs2243335	21	324kb 5' of MIR802	C	0.30	0.790	9.91E-06	1.014	0.821	1.077	0.438	1.032	0.540
rs4599120	2	4.3kb 3' of LOC84931	A	0.08	1.390	2.11E-05	0.942	0.551	0.895	0.466	0.927	0.368
rs12698902	7	AUTS2	G	0.27	1.239	3.73E-05	1.052	0.425	1.141	0.192	1.077	0.170
rs6977656	7	AUTS2	T	0.19	1.267	4.05E-05	1.065	0.375	1.109	0.358	1.077	0.214
rs2898237	21	329kb 5' of MIR802	G	0.30	0.805	4.21E-05	1.025	0.689	1.120	0.228	1.053	0.318
rs10231884	7	LOC100505881	A	0.58	0.817	4.61E-05	0.998	0.972	0.961	0.666	0.987	0.793
rs13232100	7	AUTS2	G	0.40	1.217	4.92E-05	1.036	0.544	1.130	0.197	1.062	0.233
rs11154774	6	22kb 3' of LOC154092	G	0.40	1.215	5.25E-05	1.083	0.173	1.083	0.173	1.081	0.113
rs7642234	3	DNAH12	A	0.26	1.253	6.30E-05	0.943	0.402	1.047	0.675	0.972	0.653
rs836692	2	30kb 5' of B3GALT1	T	0.32	1.225	6.44E-05	1.048	0.453	1.074	0.456	1.056	0.303
rs6852190	4	LIMCH1	T	0.36	0.813	6.88E-05	1.202	0.002	0.981	0.845	1.135	0.014
rs2063621	3	CADPS	G	0.78	0.797	6.94E-05	1.108	0.125	1.142	0.212	1.118	0.049
rs10180670	2	225kb 5' of SOX11	A	0.17	0.775	7.00E-05	0.987	0.859	0.956	0.682	0.978	0.708
rs1391412	16	294kb 3' of TERF2IP	A	0.44	0.826	8.37E-05	1.047	0.429	1.010	0.915	1.036	0.473

Table 4. Top strongest 14 SNPs ($P < 1.0\times10^{-4}$) be associated with phlegm in the GWA study.

SNPs	Chr	Gene	A1	MAF	Identification LifeLines I		Replication				Meta-analysis Replication	
							LifeLines II		Vlagtwedde-Vlaardingen			
					OR	P	OR	P	OR	P	OR	P
rs4818199	21	79kb 3' of NCRNA00323	T	0.40	0.781	3.28E-06	0.936	0.314	1.048	0.711	0.959	0.470
rs13285576	9	488kb 3' of CYLC2	A	0.16	0.724	1.68E-05	1.022	0.800	0.847	0.324	0.986	0.827
rs6828886	4	171kb 3' of PAPSS1	C	0.42	0.794	1.81E-05	0.982	0.780	1.035	0.789	0.993	0.904
rs6040994	20	30kb 3' of BTBD3	T	0.15	1.371	1.93E-05	0.930	0.481	1.055	0.794	0.954	0.607
rs9643995	8	SGCZ	T	0.03	0.471	2.79E-05	1.402	0.028	0.987	0.971	1.331	0.049
rs9831020	3	107kb 3' of LOC100507098	G	0.13	0.691	4.44E-05	1.014	0.892	0.988	0.948	1.008	0.933
rs7680755	4	230kb 3' of PAPSS1	T	0.41	0.801	4.66E-05	0.982	0.789	1.030	0.815	0.992	0.896
rs17534243	1	24kb 3' of LOC339442	G	0.21	1.265	4.91E-05	0.954	0.546	0.911	0.538	0.945	0.412
rs1156855	15	PAQR5	A	0.39	0.809	5.25E-05	1.026	0.700	1.221	0.126	1.063	0.300
rs6075458	20	145kb 5' of SLC24A3	A	0.20	0.759	5.79E-05	1.035	0.676	1.051	0.742	1.039	0.598
rs17792478	6	SENP6	G	0.33	1.233	5.92E-05	1.086	0.219	0.896	0.390	1.042	0.488
rs8025182	15	PAQR5	T	0.39	0.812	6.79E-05	1.016	0.812	1.269	0.063	1.064	0.289
rs6786757	3	SLC9A9	G	0.09	1.411	8.57E-05	1.136	0.290	1.092	0.714	1.127	0.266
rs7633390	3	27kb 5' of ZNF717	T	0.20	1.255	9.25E-05	0.970	0.691	0.994	0.967	0.975	0.710

Discussion

To the best of our knowledge, this is the first GWA study assessing genetic variants associated with cough, dyspnea, and phlegm. In the identification cohort, we identified 17, 19 and 14 SNPs associated with cough, dyspnea and phlegm respectively at a $p < 10^{-4}$ significance level. In the meta-analysis of two independent replication cohorts, one association was observed between the cough and rs16918212 located on chromosome 12 in intron of *A2MP1*, and no associations with dyspnea and phlegm were replicated.

The odds ratio for this SNP indicates that carriers of the A allele have a lower risk to cough than subjects with the wild type genotype. This SNP is located in an intron of *A2MP1* (alpha-2-macroglobulin pseudogene 1). *A2MP1* has been associated with Alzheimer's disease (24). Pseudogenes are genomic DNA sequences similar to normal genes but non-functional; they have lost their gene expression in the cell or their ability to code protein (25). Some of pseudogenes can be functional when they are transcribed. Increasing evidence suggests that pseudogenes may have important physiological functions (25).

Strengths and limitations

A major strength of this study the fact this is the first GWA study trying to identify genetic susceptibility loci for cough, dyspnea and phlegm, which included 2 verification samples: one using the same methodology (LifeLines II) and one using similar methodology (Vlagtwedde-Vlaardingen) as the discovery sample (LifeLines I). The respiratory symptoms that we studied were defined based on the standardized questionnaire of the ECRHS.

A limitation of our study might be the fact that we used a liberal p-value threshold for identification of SNPs in the discovery cohort ($p < 10^{-4}$), given the fact it is an exploratory study. When we evaluated the 50 identified SNPs further in the two independent replication cohorts we found four SNPs with a p-value < 0.05. But only one SNP showed the same direction of effect. These statistics are no better than what could be expected by chance. *A2MP1*, a pseudogene, has not been associated with lung function impairment or respiratory diseases. Therefore, this gene does not seem to be a plausible gene underlying the development of respiratory symptoms.

Conclusion

We did not find a convincing association between genetic markers and the presence of respiratory symptoms cough, dyspnea and phlegm. The lack of finding a plausible significant association between SNPs and respiratory symptoms can be explained by the fact that a respiratory symptom can be caused by different environmental exposures or can be a presentation of different underlying diseases with specific genetic or environmental origins. Susceptibility to these various exposures may be genetically determined and susceptibility loci may differ between exposures. The environment should thus be taken into account when studying the association between genetics and respiratory symptoms. Therefore, the next logical step will be performing a genome-wide interaction (GWI) study to identify genetic loci for respiratory symptoms in interaction with known harmful environmental exposures.

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Abbreviations

SNP: Single nucleotide polymorphism

GWAS; Genome Wide Association Study

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CHAPTER 6

Genome-wide interaction study of gene-by-occupational exposures on respiratory symptoms

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Abstract

Rationale:

Respiratory symptoms such as cough, dyspnea, and phlegm are important indicators of respiratory diseases and contribute to hospitalizations and lung function decline. Both genetic and environmental factors contribute to the development of respiratory symptoms. Less is known about effects of gene-environment interaction on respiratory symptoms.

Objectives：

The current study aimed to assess single nucleotide polymorphisms (SNPs)-by-occupational exposure interaction effects on the respiratory symptoms cough, dyspnea and phlegm.

Methods:

We performed an initial analysis in the first data release sample (LifeLines I) from the LifeLines cohort study (n=7,976 subjects). Job-specific exposure was estimated with the ALOHA+job exposure matrix. Effects of SNP-by-occupational exposure interaction on respiratory symptoms were tested using a logistic regression model adjusted for gender, age, and current smoking. SNPs with a SNP-by-occupational exposure interaction p-value < 10^{-4} were replicated in the second LifeLines data release sample (LifeLines II; n=5,260) and the Vlagtwedde-Vlaardingen cohort study (n=1,529) using meta-analysis. Additionally, we assessed whether replicated SNPs influenced gene expression by analyzing if they were cis-acting expression (mRNA) quantitative trait loci (eQTL) in lung tissue.

Results:

A total of 24 out of 599 SNPs-by-occupational exposure interactions were significantly replicated and 11 of these 24 interactions were in the same direction in all cohorts. Several of these loci were plausible candidates for respiratory symptoms, given their important function in the lungs, such as *TMPRSS9*, *TOX3*, and *ARHGAP18*. Three of 11 replicated SNPs were cis-acting eQTL associated with gene expression of *FCER1A, CHN1* and *TIMM13* in lung tissue.

Conclusions

This genome-wide interaction study in the context of occupational exposures in relation to cough, dyspnea, and phlegm identified several susceptibility genes. Further research should investigate the function of the identified genes in relation to occupational exposures.

Introduction

Occupational exposures have been associated with low levels of lung function, high risk for respiratory symptoms and COPD (1-4). Respiratory symptoms in adults are related to impaired quality of life regardless of asthma and COPD (5). Previous candidate gene approaches have shown that genetic variants may affect the level of lung function (6) and the risk for respiratory symptoms (7). For example polymorphisms in *ADAM33* were associated with decline in lung function and genetic polymorphisms in *TLR4* were associated with work-related respiratory symptoms (8, 9). Clearly, these candidate gene studies were driven by hypotheses relying on known biological pathways. Contrary to that, a genome-wide association study (GWAS) can identify genetic loci across the entire genome in a hypothesis free manner. However, most single GWASes do not replicate consistently, which might be caused by environmental factors that trigger the development of symptoms only in subjects with a specific genotype. More recently, hypothesis free genome-wide interaction studies (GWIS) were performed to identify genetic loci that affect the susceptibility to known harmful exposures (10-12). We have recently performed a GWAS on respiratory symptoms, and found no significant associations between genetic loci and respiratory symptoms including cough, dyspnea and phlegm (paper submitted). This strengthens the suggestion that genetic determinants may play an important role only in combination with specific environmental exposures such as occupational exposures. Therefore, it is necessary to investigate genetic susceptibility to environmental exposure in relation to respiratory symptoms using a GWIS.

We have previously shown that genetic susceptibility is associated with lung function level (FEV_1) in the context of occupational exposure to biological dust, mineral dust and gases and fumes, and that several of these novel identified susceptibility loci were cis-acting eQTLs in lung

tissue (10). Whether such gene-by-occupational exposure interactions exist in relation to respiratory symptoms is largely unknown.

The aim of the current GWIS was to identify susceptibility loci for several types of occupational exposures in relation to the respiratory symptoms cough, dyspnea, and phlegm in a general population cohort. We used two independent cohorts to confirm our findings. This may shed light on the genetic susceptibility to specific exposures leading to respiratory symptoms.

Methods

Identification cohort

Genotyped individuals from the first data release of the LifeLines cohort study (LifeLines I) with full data on all covariates were included (n = 7,976). The LifeLines cohort study is a general population based cohort that started in 2006, including subjects from the three Northern provinces of the Netherlands (10, 13). Participants completed a standardized questionnaire, and provided written informed consent. The study protocol was approved by the local medical ethics committee.

Replication cohorts

To verify our initial findings, we included 5,260 subjects from the second data release of the LifeLines cohort study (LifeLines II) and 1,529 subjects with full data on genotypes and covariates on the last survey in 1989/1990 from the Vlagtwedde-Vlaardingen cohort, a prospective general population based cohort (8, 14). Both the identification and replication cohorts included only Caucasian individuals of Dutch descent.

Genotyping and quality control

Blood samples of all subjects included in the identification and replication cohorts were genotyped using IlluminaCytoSNP-12 arrays. Samples with call rates < 95% were excluded. SNPs that fulfilled the quality control

criteria were included: genotype call-rate ≥ 95% (on average a call-rate of > 99% is achieved), minor allele frequency (MAF) ≥ 1%, and Hardy-Weinberg equilibrium (HWE) cut-off p-value ≥ 10^{-4}. A total of 227,981 genotyped SNPs were included in the identification analysis. Non-Caucasian samples and first-degree relatives were excluded.

Respiratory symptoms

Cough, dyspnea, and phlegm were defined by a standardized questionnaire from the European Community Respiratory Health Survey (ECRHS) in LifeLines I and LifeLines II (15) and the British Medical Research Council (BMRC) respiratory questionnaire in Vlagtwedde-Vlaardingen cohort (16). Cough was defined as at least one positive answer to the questions: “do you usually cough first thing in the morning in the winter?” or “do you usually cough during the day, or at night, in winter?”. Dyspnea was defined as a positive answer to the question: “are you troubled by shortness of breath when hurrying on level ground or walking up a slight hill or stairs at normal pace?”. Phlegm was defined as at least one positive answer to the questions: “do you usually bring up any phlegm from your chest first thing in the morning in winter?” or “do you usually bring up any phlegm from your chest during the day, or at night, in winter?”.

Occupational exposure

Self-reported job title and descriptions were coded according to the International Standard Classification of Occupations version 1988 (ISCO-88) (10, 17). These four-digit codes were used to estimate job-specific exposure to vapors, gas, dust or fumes (VGDF), biological dust, mineral dust, gas and fumes, pesticides, herbicides, insecticides, aromatic solvents, chlorinated solvents, other solvents, and metals using the ALOHA+ Job Exposure Matrix (JEM) (1, 18). The ALOHA+ JEM classifies subjects based on the ISCO-88 job codes into no, low and high exposure. In this study, “no exposure” was recoded to “0”, and both “low exposure” and “high exposure” were recoded to “1”.

Statistical analysis

The data are presented as median (min-max) for continuous variables and as frequencies (percentages) for categorical variables. SNPs were tested in an additive genetic model. Effects of gene-environment interactions on the respiratory symptoms cough, dyspnea, and phlegm were tested by including the SNP, the occupational exposure and the SNP-by-occupational exposure interaction term in a logistic regression model adjusted for sex, age, and current smoking (no/yes) in the software package PLINK (version 1.07) (19). SNPs with a SNP-by-occupational exposure interaction p-value < 10^{-4} were taken forward for replication in the two independent cohorts. The interaction estimates of the two replication cohorts were meta-analyzed using PLINK. Replication was achieved when the p-value of the meta-analysis was < 0.05 and the interaction effect was in the same direction as in the identification cohort. SNP annotation was performed using HaploReg version 4.1 (Broad Institute) (20).

Gene expression analysis

We assessed whether the replicated SNPs were cis-acting expression (mRNA) quantitative trait loci (eQTLs) in a lung tissue database established by the lung eQTL consortium (21). Lung tissue was collected from patients who underwent lung resectional surgery at three participating sites; University of Groningen, Laval University, and University of British Columbia (21). DNA samples were genotyped with Illumina Human1M-Duo BeadChip arrays, and gene expression profiles were obtained using a custom Affymetrix array (GEO accession number GPL10379 and GSE23546). A total of 1,087 subjects with no missing data were included in the analysis. The association between the SNPs and gene expression levels (log transformed) were tested in each cohort separately by linear regression analysis adjusted for disease status, age, gender, smoking status and a cohort specific number of principal components, followed by a meta-analysis of the results of the 3 cohorts. A cis-eQTL was defined as a SNP that was significantly associated with expression levels of a gene within a 1 Mb (in both directions) distance of

that SNP, with a p-value below the Bonferroni corrected threshold (p = 0.05/number of probe sets within the 2 Mb window) in the meta-analysis.

Results

Characteristics of the study populations and the prevalence of exposures and respiratory symptoms are shown in Table 1. The prevalence of smoking, occupational exposures and respiratory symptoms were higher in the Vlagtwedde-Vlaardingen cohort compared to LifeLines I and LifeLines II. The genomic inflation factor for the identification sample suggests little population stratification for SNP-by-occupational exposures interaction models such as SNP-by-biological dust exposure on cough, SNP-by-mineral dust exposure on dyspnea and SNP-by-aromatic solvent exposure on phlegm (λ = 0.99, 1.01, 1.02, respectively, online supplement Figure S1).

SNP by exposures interactions

Cough

We identified 205 SNPs that interacted with the investigated occupational exposures on cough in the identification cohort (p-values for interaction < 10^{-4}) (table 2). In total 8 SNP-by-exposure interactions (3.9%) had a p <0.05 in the meta-analysis of the replication cohorts, of which 2 interactions were in the same direction as in the identification cohort (Figure1, Table 2 and Table 3).

Table 1. Characteristics of the subjects included in the identification (LifeLines I) and replication (LifeLines II and Vlagtwedde-Vlaardingen) cohorts.

	Identification	Replication	
	LifeLines I	LifeLines II	Vlagtwedde-Vlaardingen
N*	7,976	5,260	1,529
Male, n (%)	3420 (43)	2112 (40)	714 (46)
Age (yrs), median (min-max)	48.02 (20-89.0)	47.7 (18-90)	51.0 (35-65)
Current smokers, n (%)	1904 (24)	1051 (20)	548 (36)
Smoking status			
Ever, n (%)	4,737 (59)	3,050 (58)	1,054 (69)
Never, n (%)	3,239 (41)	2,209 (42)	475 (31)
Respiratory symptoms, n (%)			
Cough	675 (9)	409 (8)	175 (12)
Dyspnea	547 (7)	358 (7)	147 (10)
Phlegm	1702 (21)	1068 (20)	337 (25)
Exposure, n (%)			
VGDF	3580 (45)	2482 (47)	972 (64)
Biological dust	2467 (31)	1777 (34)	696 (46)
Mineral dust	1709 (21)	1177 (22)	595 (39)
Gases and fumes	3285 (41)	2252 (43)	887 (58)
Pesticides	309 (4)	251 (5)	292 (19)
Herbicides	108 (1)	98 (2)	233 (15)
Insecticides	266 (3)	229 (5)	272 (18)
Solvents			
Aromatic solvents	748 (9)	465 (9)	402 (26)
Chlorinated solvents	598 (7)	397 (8)	189 (12)
Other solvents	1812 (23)	1241 (24)	362 (24)
Metals	580 (7)	336 (6)	163 (11)

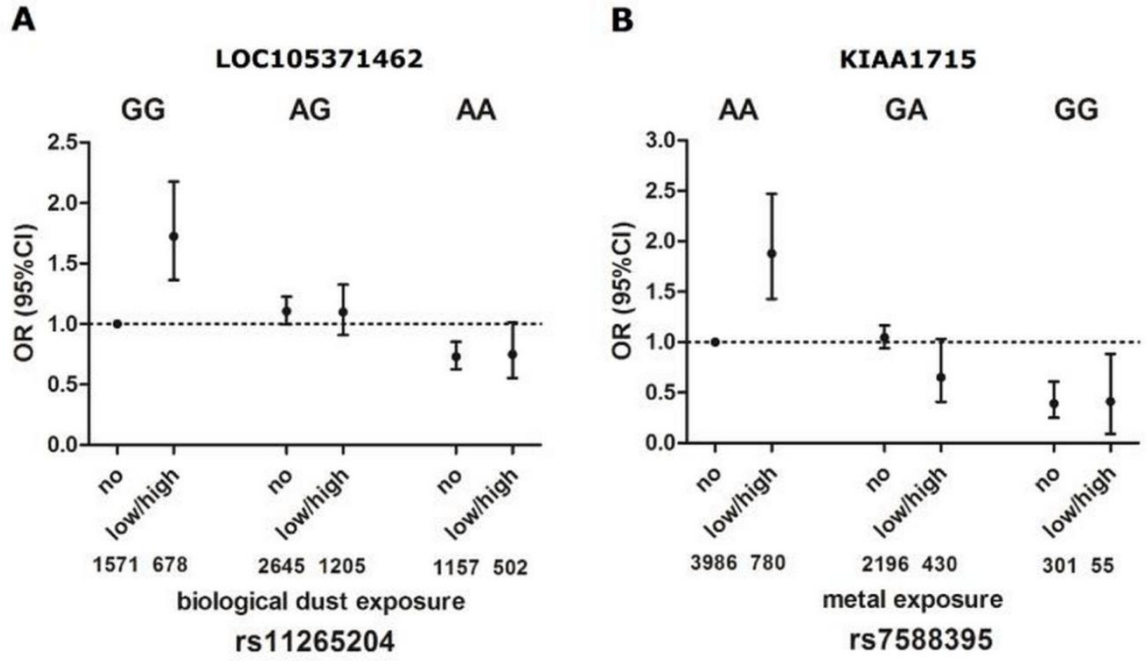

Figure 1. Associations between occupational exposure and cough stratified by genotype in the identification cohort.

Table 2. Flow table showing the selection of SNPs for cough, dyspnea and phlegm.

	N SNPs Identification (LifeLines I)	N SNPs Replication (Meta-analysis of LifeLines II and Vlagtwedde-Vlaardingen)	
Cough	**p value < 10^{-4}**	**p value < 0.05**	**same direction**
Total	205	8	2
VGDF	27	3	
Biological dust	25	1	1
Mineral dust	14		
Gases and fumes	25	2	
Pesticides	13		
Herbicides	3		
Insecticides	14		
Solvents			
Aromatic solvents	14		
Chlorinated solvents	31	1	
Other solvents	16		
Metals	23	1	1
Dyspnea	**p value < 10^{-4}**	**p value < 0.05**	**same direction**
Total	220	12	6
VGDF	18	1	1
Biological dust	14		
Mineral dust	30	6	3
Gases and fumes	16	1	1
Pesticides	27		
Herbicides	1		
Insecticides	20	2	
Solvents			

Aromatic solvents	28		
Chlorinated solvents	24		
Other solvents	29	1	
Metals	13	1	1
Phlegm	**p value < 10^{-4}**	**p value < 0.05**	**same direction**
Total	174	4	3
VGDF	20		
Biological dust	20	2	1
Mineral dust	11		
Gases and fumes	23		
Pesticides	6		
Herbicides	4		
Insecticides	6		
Solvents			
Aromatic solvents	29	1	1
Chlorinated solvents	22		
Other solvents	20	1	1
Metals	13		

146 **Table 3**. Associations between SNPs and cough in the identification cohort and the replication meta-analysis.

SNPs	**Chr**	**A1**	**MAF**	**Closest gene**	**LifeLines I Identification (n=7976)**		**LifeLines II Replication (n=5260)**		**Vla-Vla Replication (n=1529)**		**Meta-analysis Replication (n=6806)**	
rs11265204	**1**	**A**	**0.32**	**LOC105371462 (intronic)**	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**	**P**
SNP					1.10	6.50×10^{-2}	1.05	4.54×10^{-1}	1.20	2.72×10^{-1}	1.08	2.65×10^{-1}
Biological dust					1.69	1.01×10^{-6}	1.29	5.80×10^{-2}	1.78	3.60×10^{-2}	1.38	9.00×10^{-3}
SNP* biological dust					0.66	1.47×10^{-5}	0.82	9.40×10^{-2}	0.64	5.60×10^{-2}	0.78	1.90×10^{-2}
rs7588395	**2**	**A**	**0.23**	**KIAA1715 (300 kb 3')**	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**	**P**
SNP					1.86	5.26×10^{-6}	1.12	9.30×10^{-2}	1.01	9.45×10^{-1}	1.10	1.17×10^{-1}
Metals					1.05	4.21×10^{-6}	1.50	3.40×10^{-2}	0.99	9.89×10^{-1}	1.34	7.20×10^{-2}
SNP*metals					0.38	2.31×10^{-5}	0.60	5.10×10^{-2}	0.64	4.10×10^{-1}	0.61	3.50×10^{-2}

Dyspnea

We identified 220 SNPs that interacted with the investigated occupational exposures on dyspnea in the identification cohort (p-values for interaction < 10^{-4}) (Table 2). In the meta-analysis of the replication cohorts, interactions with 12 SNPs (5.5%) had a p <0.05 of which 6 interactions were in the same direction as in the identification cohort (Figure2, Table 2 and Table 4).

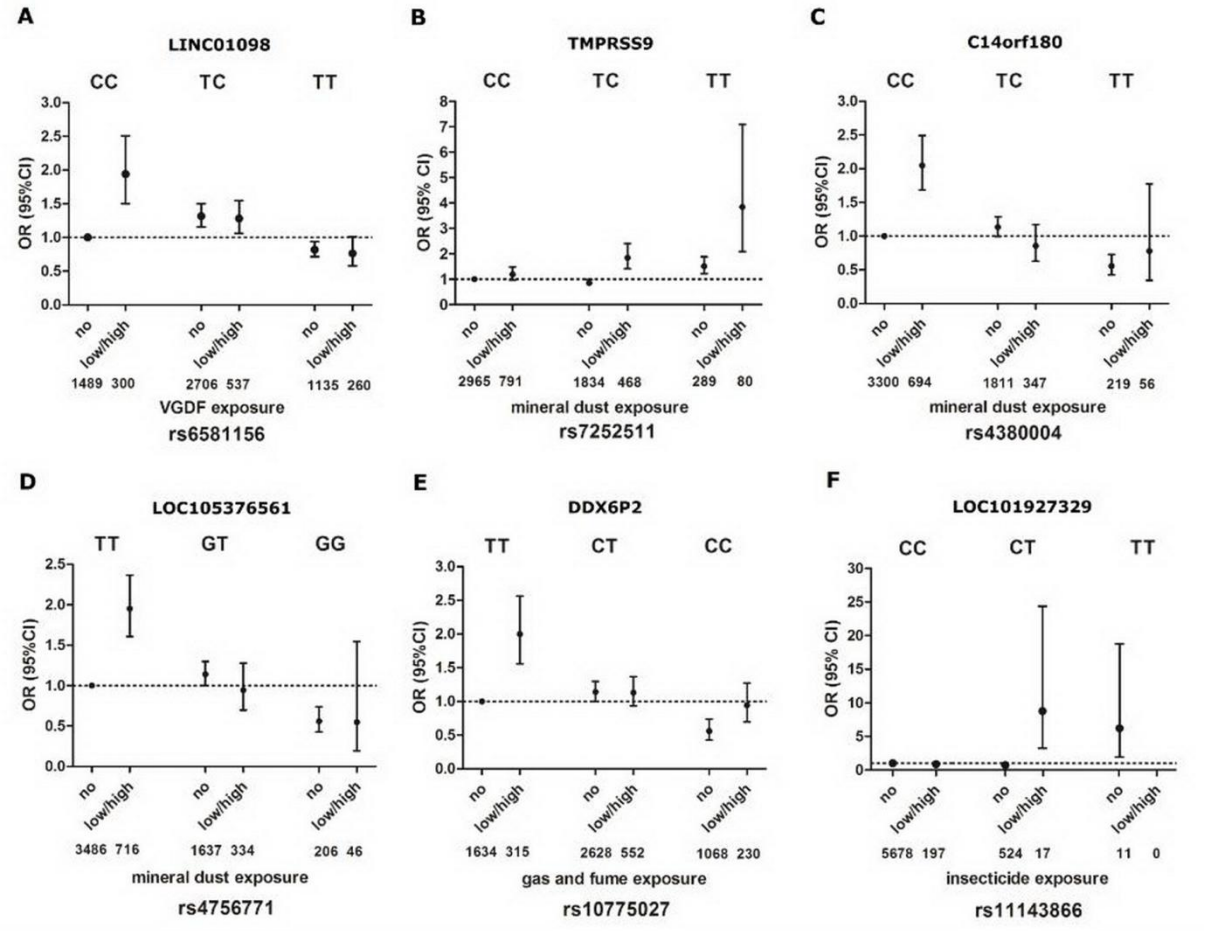

Figure 2. Associations between occupational exposure and dyspnea stratified by genotype in the identification cohort.

148 **Table 4**. Associations between SNPs and dyspnea in the identification cohort and the replication meta-analysis.

SNPs	**Chr**	**A1**	**MAF**	**Closest gene**	**LifeLines I Identification (n=7976)**		**LifeLines II Replication (n=5260)**		**Vla-Vla Replication (n=1529)**		**Meta-analysis Replication (n=6806)**	
rs6851156	**4**	**A**	**0.32**	**LINC01098 (intronic)**	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**	**P**
SNP					1.32	3.31×10^{-5}	1.15	7.40×10^{-2}	1.20	2.28×10^{-1}	1.16	3.20×10^{-2}
VGDF					2.01	1.20×10^{-9}	1.43	1.10×10^{-2}	1.45	1.17×10^{-1}	1.28	6.50×10^{-2}
SNP*VGDF					0.62	8.22×10^{-7}	0.77	2.50×10^{-2}	0.87	4.61×10^{-1}	0.80	2.20×10^{-2}
rs7252511	**19**	**T**	**0.30**	**TMPRSS9 (intronic)**	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**	**P**
SNP					0.85	1.34×10^{-2}	0.91	2.43×10^{-1}	0.65	4.00×10^{-3}	0.85	1.90×10^{-2}
Mineral dust					1.13	2.36×10^{-1}	1.30	3.70×10^{-2}	0.76	1.12×10^{-1}	1.08	4.58×10^{-1}
SNP*mineral dust					1.78	6.69×10^{-6}	1.22	2.19×10^{-1}	1.93	4.00×10^{-3}	1.41	8.00×10^{-3}
rs4380004	**14**	**T**	**0.18**	**C14orf180 (69kb 5')**	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**	**P**
SNP					1.11	5.60×10^{-2}	0.89	1.68×10^{-1}	1.18	2.44×10^{-1}	0.96	5.50×10^{-2}
Mineral dust					1.98	2.83×10^{-12}	1.54	3.22×10^{-4}	1.25	1.75×10^{-1}	1.43	2.11×10^{-4}
SNP*mineral dust					0.49	2.37×10^{-6}	0.81	2.25×10^{-1}	0.59	2.40×10^{-2}	0.72	2.00×10^{-2}
rs4756771	**11**	**A**	**0.25**	**LOC105376561 (intronic)**	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**	**P**
SNP					1.14	4.47×10^{-2}	1.09	2.72×10^{-1}	1.19	2.33×10^{-1}	1.12	1.23×10^{-1}
Mineral dust					1.94	6.84×10^{-12}	1.65	2.64×10^{-5}	1.17	3.33×10^{-1}	1.46	7.16×10^{-5}

SNP*mineral dust					0.49	5.68×10^{-6}	0.67	2.70×10^{-2}	0.68	1.09×10^{-1}	0.61	6.41×10^{-3}
rs10775027	**13**	**C**	**0.44**	**DDX6P2 (117 kb 3')**	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**	**P**
SNP					1.25	5.38×10^{-4}	1.10	2.04×10^{-1}	1.07	6.07×10^{-1}	1.10	1.73×10^{-1}
Gases and fumes					1.82	7.42×10^{-8}	1.32	3.80×10^{-2}	1.57	3.10×10^{-2}	1.39	4.00×10^{-3}
SNP*gases and fumes					0.68	8.87×10^{-5}	0.86	1.91×10^{-1}	0.71	5.40×10^{-2}	0.81	3.20×10^{-2}
rs11143866	**9**	**C**	**0.08**	**LOC101927329 (intronic)**	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**	**P**
SNP					0.75	2.30×10^{-2}	1.08	5.80×10^{-1}	1.25	2.96×10^{-1}	1.03	8.13×10^{-1}
Insecticides					0.88	5.38×10^{-1}	0.97	8.78×10^{-1}	0.78	1.87×10^{-1}	1.02	9.09×10^{-1}
SNP*insecticides					8.89	7.11×10^{-5}	5.48	7.00×10^{-3}	1.74	2.67×10^{-1}	2.70	1.10×10^{-2}

Phlegm

We identified 174 SNPs that interacted with the investigated occupational exposures on phlegm in the identification cohort (p-values for interaction < 10^{-4}) (Table 2). A total 4 SNP-by-occupational exposure interactions (2.3%) showed a significant association (p <0.05) in the meta-analysis of the replication cohorts, of which 3 interactions were in the same direction as in the identification cohort (Figure3, Table 2 and Table 5).

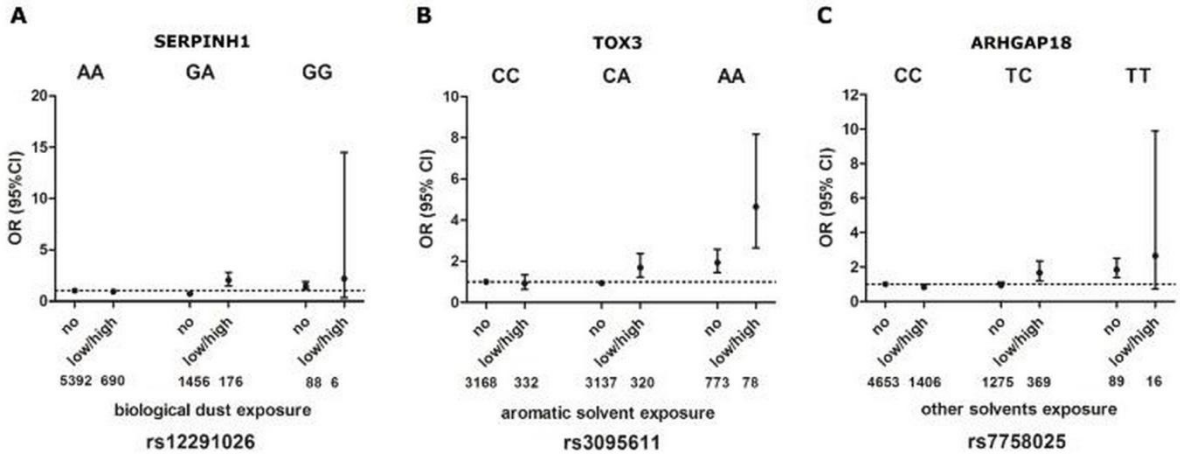

Figure 3. Associations between occupational exposure and phlegm stratified by genotype in the identification cohort

Table 5. Associations between SNPs and phlegm in the identification cohort and the replication meta-analysis.

SNPs	**Chr**	**A1**	**MAF**	**Closest gene**	**LifeLines I Identification (n=7976)**		**LifeLines II Replication (n=5260)**		**Vla-Vla Replication (n=1529)**		**Meta-analysis Replication (n=6806)**	
rs12291026	**11**	**C**	**0.08**	**SERPINH1 (18kb 5')**	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**	**P**
SNP					0.71	1.71×10^{-2}	0.85	2.24×10^{-1}	0.90	7.07×10^{-1}	0.86	2.07×10^{-1}
Biological dust					0.94	4.61×10^{-1}	1.05	6.82×10^{-1}	0.95	7.81×10^{-1}	1.02	8.24×10^{-1}
SNP*biological dust					2.03	4.03×10^{-5}	1.45	6.80×10^{-2}	0.95	7.81×10^{-1}	1.44	4.60×10^{-2}
rs3095611	**16**	**A**	**0.32**	**TOX3 (intronic)**	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**	**P**
SNP					0.94	2.73×10^{-1}	0.81	4.00×10^{-3}	0.81	1.99×10^{-1}	0.81	2.00×10^{-3}
Aromatic solvents					0.89	4.93×10^{-1}	0.66	7.80×10^{-2}	0.69	2.06×10^{-1}	0.68	3.00×10^{-2}
SNP*aromatic solvents					2.08	4.54×10^{-6}	1.65	2.90×10^{-2}	0.69	2.06×10^{-1}	1.61	8.00×10^{-3}
rs7758025	**6**	**A**	**0.32**	**ARHGAP18 (intronic)**	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**	**P**
SNP					0.94	5.03×10^{-1}	0.84	1.59×10^{-1}	1.09	6.98×10^{-1}	0.90	2.97×10^{-1}
Other solvents					0.82	5.20×10^{-2}	0.88	2.93×10^{-1}	1.01	9.62×10^{-1}	0.90	3.65×10^{-1}
SNP*other solvents					2.02	7.15×10^{-7}	1.55	5.80×10^{-2}	1.36	4.58×10^{-1}	1.51	4.40×10^{-2}

Expression Quantitative Trait Loci (eQTL)

Of the 11 replicated SNPs, 7 SNPs were available in the lung tissue database and for 2 other SNPs a SNP in high LD could be found (i.e. rs11682476 for rs7588395: r^2=0.99 and rs1420546 for rs3095611: r^2=0.93 (20)), 2 SNPs (rs6851156 and rs4380004) could not be assessed for their association with gene expression levels in lung tissue. Three SNPs showed cis-eQTL associations with p-values below the Bonferroni corrected threshold (Table S1). SNP rs11265204 that significantly interacted with exposure to biological dust on cough was associated with expression of *FCER1A* ($p = 1.99 \times 10^{-10}$) (Figure 4A). Rs11682476 (proxy SNP for rs7588395) significantly interacted with exposure to biological dust on cough was associated with expression of *CHN1* ($p = 1.79 \times 10^{-4}$) (Figure 4B). SNP rs7252511 that interacted with exposure to mineral dust on dyspnea was significantly associated with expression of *TIMM13* (close to *TMPRSS9*) ($p = 1.27 \times 10^{-8}$) (Figure 4C).

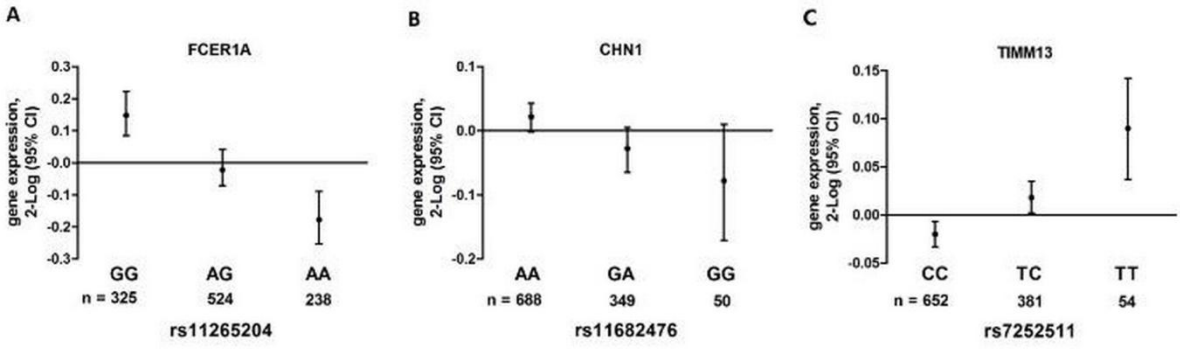

Figure 4. Gene expression levels stratified by genotype for cis-eQTL SNPs.

Discussion

To our knowledge this is the first GWIS focusing on the effects of SNPs-by-occupational exposure interactions on respiratory symptoms. We identified and replicated two SNPs that were associated with cough in subjects with occupational exposure to biological dust and metals, respectively. Six SNPs were identified and replicated in SNP-by-occupational exposure interaction analysis on dyspnea in subjects with exposure to VGDF, mineral dust, gases and fumes, and insecticides. Three SNPs were associated with phlegm in subjects with exposure to biological dust, aromatic solvents, and other solvents, respectively. Several identified SNPs were located in genes that have important functions in the lung and may play a role in pathways associated with respiratory symptoms (i.e. *TMPRSS9, TOX3, ARHGAP18,* and *FCER1A*) (Table S2). In addition, three SNPs were associated with gene expression levels in the lung which indicate their possible functional relevance.

Findings of the identification cohort were replicated in two independent cohorts. The occupational exposure definition was the same in all cohorts, and estimated by the ALOHA+ JEM that is specifically designed for population-based studies (22), and less likely to be affected by recall bias or differential misclassification when compared to self-report (23). We investigated a broad range of occupational exposures, being VGDF, biological dust, mineral dust, gases and fumes, pesticides, herbicides, insecticides, aromatic solvents, chlorinated solvents, other solvents, and metals, related to the respiratory symptoms cough, dyspnea and phlegm.

The minor allele of rs7252511, a SNP in *TMPRSS9*, was associated with a higher risk of dyspnea in subjects exposed to mineral dust. In lung tissue this SNP rs7252511 showed a cis-eQTL association with higher expression of the gene *TIMM13,* which partially overlaps in antisense orientation with *TMPRSS9*, and may have a similar function as *TMPRSS9* in the lung (24). *TMPRSS9*, known as polyserase-1, is a type II transmembrane serine protease and has a unique structure including

three tandem serine protease domains (i.e., serase-1, -2, and -3) (25). Serase-1B is known to interact with glycosaminoglycans (GAGs), which maintain lung structure and function modulate the inflammatory response, influence tissue repair and remodeling, and protect against injury in various respiratory diseases (25-27). Serase-1B and urokinase-type plasminogen activator (uPA) receptor can efficiently activate uPA, which is involved in epithelial repair and airway remodeling, and links with asthma susceptibility, bronchial hyperresponsiveness, and decline in lung function (28-30). Occupational exposure to mineral dust is a well-known risk factor for reduced lung growth and COPD (31-34) of which dyspnea is one of the main symptoms. Our results demonstrate that subjects carrying the minor allele of this SNP in *TMPRSS9* are especially vulnerable to these harmful effects of mineral dust exposure

Another interesting finding is the association between rs3095611, a SNP in *TOX3*, and a higher risk of phlegm in subjects exposed to aromatic solvents. *TOX3* is a neuronal survival factor that regulates calcium dependent transcription in neurons (35). *TOX3* contains a nuclear localization signal and a high-mobility group (HMG) box domain, indicating that it may be involved in bending and unwinding of DNA and alteration of chromatin structure (36-38). In addition, *TOX3* is mediating inflammatory responses in the lung (35-39). Our results indicate there is interaction between *TOX3* and exposure to aromatic solvents regarding the risk of phlegm. When aromatic solvents enter the body by inhalation, they can cause respiratory inflammatory, upper respiratory tract damage, and, interestingly, chronic bronchitis, with phlegm as an important symptom (40-43). Our results suggest that subjects carrying the minor alleles of this SNP in TOX3 are more susceptible to the respiratory effects of exposure to aromatic solvents.

Exposure to other solvents (i.e. non-aromatic solvents such as formaldehyde and dimethylformamide) is a risk factor for phlegm in subjects carrying the minor allele of rs7758025 in the *ARHGAP18* gene. *ARHGAP18* regulates the integrity and morphogenesis of epithelial cells

(44,45). The epithelial cells in lungs provide structural integrity, moisten and protect the airways, are a physical barrier against environmental exposures, and prevent infection and tissue injury by action of the mucociliary escalator (46,47). Genetic variation in the *ARHGAP18* gene may thus result in an impaired or altered epithelial barrier function, which might lead to increased phlegm production upon exposure to non-aromatic solvents.

The cis-eQTL analysis showed three additional associations that are of interest. The first was an association between the intergenic SNP rs11265204 and a lower expression of *FCER1A*. In our analysis, the interaction between rs11265204 and biological dust was significantly associated with a lower risk to cough. Polymorphisms in *FCER1A* are associated with IgE levels and allergic sensitization (48-50), and therefore, speculations are that *FCER1A* is associated with asthma via IgE (51, 52). Given that cough is a symptom associated with asthma and allergy (53), our results indicating a lower expression of *FCER1A* and a lower risk of exposure-associated coughing in subjects carrying the minor alleles of rs11265204 thus seems plausible. The second cis-eQTL is rs11682476 (proxy-SNP for rs7588395) which is associated with lower expression of *CHN1*. According to our analysis, the interaction between rs7588395 and metal exposure was significantly associated with cough. *CHN1* encodes α2-chimaerin which is a non-protein kinase C phorbol ester receptor with Rac-GTPase-activating protein activity and cysteine-rich domain (CRD). Ahmed et al demonstrated that cysteine-rich proteins indicate the presence of metal-binding domains, where metals, usually zinc, play a structural role in maintaining the function of these domains, such as dimer formation and DNA-binding (54). In the current study, we found that subjects with the major allele of rs7588395 and exposure to metals had increased prevalence of cough. An important paralog of this gene is *ARHGAP18* which belongs, like *CHN1*, to a family of Rho GTPase-activating proteins. Therefore, *CHN1* may have a similar function in the lung as *ARHGAP18.* The third cis-eQTL is rs7252511 associated with higher expression of gene *TIMM13* with some overlap in

sequence with *TMPRSS9,* and may play a similar important role as *TMPRSS9* does in the lung (24).

Since GWISes are an explorative approach, we used a more liberal p-value threshold ($p < 10^{-4}$) for identification of SNPs in the identification cohort to keep the risk low of not detecting a true association between SNP-by-occupational exposure interactions and respiratory symptoms. When we assessed these interactions in the independent replication cohorts, the total number of significant gene-by-exposure interactions in the replication meta-analysis is less than expected by chance (i.e. 24 out of 599 (approximately 4.1%) had a p-value < 0.05). However, given the putative function of the identified genes and the association with gene expression levels we think the associations may be true findings. Further research is necessary to confirm this.

In conclusion, this is the first GWIS on the risk of respiratory symptoms cough, dyspnea and phlegm, which investigated interactions between SNPs and several types of occupational exposures. We identified some plausible candidate genes that may be involved in biological pathways leading to respiratory symptoms, i.e. *FCER1A, CHN1, TMPRSS9, TOX3,* and *ARHGAP18*. The next step should be to determine if the identified genes are true susceptibility loci for respiratory symptoms. This study may eventually contribute to the understanding of pathways underlying the development of respiratory symptoms.

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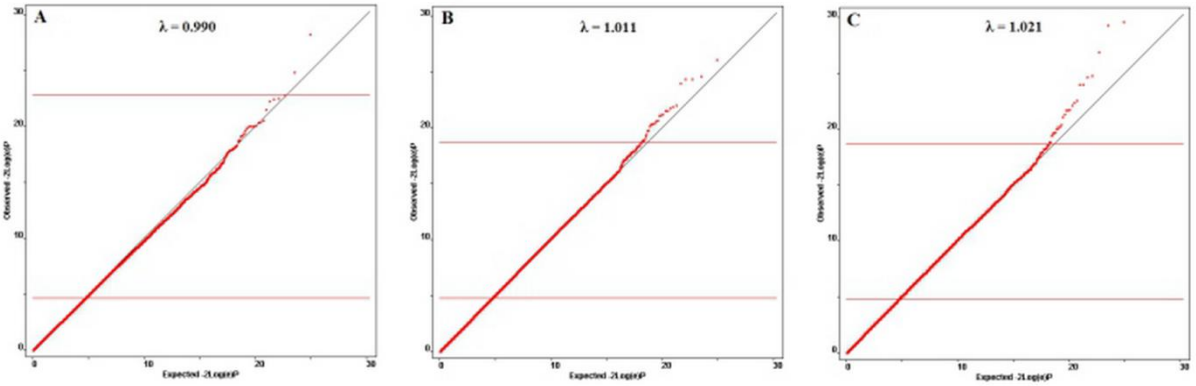

Figure S1. QQ-plots for the marginal association of the SNP-by-biological dust exposure on cough (A), SNP-by-mineral dust exposure on dyspnea (B) and SNP-by-aromatic solvent exposure on phlegm (C) in the identification samples, adjusted for sex, age and current smoking.

Table S1. Results of the gene expression analysis in lung tissue for replicated SNPs (GWIS_Occupational exposures).

SNP	Symptoms	Analysis	Probe set	Gene	B	SE	p-value	Threshold	Effect alle	Referenc e alle
rs11265204	Cough	Biological dust	100138809_TGI_at	*FCER1A*	-0.17	0.03	1.99×10^{-10}	5.05×10^{-4}	G	A
rs11682476	Cough	Metals	100304521_TGI_at	*CHN1*	-0.05	0.01	1.79×10^{-4}	1.06×10^{-3}	A	G
			100306391_TGI_at	*CHN1*	-0.06	0.01	2.36×10^{-4}			
rs7252511	Dyspnea	Mineral dust	100149721_TGI_at	*TIMM13*	0.04	0.01	1.27×10^{-8}	4.13×10^{-4}	C	T

SE = standard error, threshold was calculated as p value = 0.05/number probes with 2 Mb window. Rs11682476 and rs7588395 were in strong LD (r^2 = 0.99).

164 Table S2. Putative function of the identified and replicated genes.

SNP	Chr	Nearest gene	Symptoms	Putative function of nearest gene*
rs11265204	1	LOC105371462 (intronic)	Cough	LOC105371462 (no-coding RNA Gene) is affiliated with the ncRNA class. SNP rs11265204 was associated with asthma#.
rs7588395	2	KIAA1715 (300kb 3')	Cough	KIAA1715 is a Protein Coding gene. Plays a role in tubular endoplasmic reticulum network formation and maintenance. May be involved in limb and central nervous system development.
rs6851156	4	LINC01098 (intronic)	Dyspnea	LINC01098 (no-coding RNA Gene) is affiliated with the non-coding RNA class.
rs7252511	19	TMPRSS9 (intronic)	Dyspnea	TMPRSS9 (transmembrane serine protease 9)/ Polyserase-1 (polyserine protease-1)) is a Protein Coding gene. GO annotations related to this gene include serine-type endopeptidase activity.
rs4380004	14	C14orf180 (69kb 5')	Dyspnea	C14orf180, known as Nrac, is a nutritionally-regulated adipose and cardiac-enriched gene. Nrac is expressed specifically and abundantly in fat and the heart.
rs4756771	11	LOC105376561 (intronic)	Dyspnea	LOC105376561 (no-coding RNA Gene) is affiliated with the ncRNA class.
rs10775027	13	DDX6P2 (117kb 3')	Dyspnea	DDX6P2 (DEAD (Asp-Glu-Ala-Asp) Box Helicase 6 Pseudogene 2) is a Pseudogene.
rs11143866	9	LOC101927329 (intronic)	Dyspnea	LOC101927329 (no-coding RNA Gene) is affiliated with the ncRNA class. SNP rs11143866 was associated with serum total immunoglobulin E (IgE)#.
rs12291026	11	SERPINH1 (18kb 5')	Phlegm	SERPINH1 (Serpin Peptidase Inhibitor, Clade H (Heat Shock Protein 47), Member 1, (Collagen Binding Protein 1)). Among its related pathways are Endochondral Ossification and Degradation of the extracellular matrix. GO annotations related to this gene include unfolded protein binding and collagen binding.

rs3095611	16	TOX3 (intronic)	Phlegm	TOX3 (TOX High Mobility Group Box Family Member 3) is a Protein Coding gene. GO annotations related to this gene include protein homodimerization activity and phosphoprotein binding; it may be involved in bending and unwinding of DNA, alteration of chromatin structure, and regulation of calcium-dependent transcription and interacts with cAMP-response-element-binding protein.
rs7758025	6	ARHGAP18 (intronic)	Phlegm	Rho GTPase activating protein 18 (ARHGAP18) is a Protein Coding gene. Among its related pathways are Signaling by GPCR and Rho GTPase cycle. GO annotations related to this gene include GTPase activator activity.

*: information obtained from GeneCards website (http://www.genecards.org/).

#: information obtained from GWASCENTRAL website (http://www.gwascentral.org/)

Table S3. SNPs acting as cis-eQTLs on genes within a 1 Mb distance.

SNP	Number of tested probes	Number of significantly associated probes
Cough		
rs11265204	99	1
rs7588395/rs11682476	47	2
Dyspnea		
rs7252511	121	1
rs11143866	18	0
rs4380004/rs4353434	0	0
rs6851156	0	0
rs10775027	1	0
rs4756771	33	0
Phlegm		
rs3095611/rs1420546	21	0
rs7758025	16	0
rs12291026	53	0

Rs11682476 and rs7588395 were in strong LD (r^2 = 0.99); rs4353434 and rs4380004 were in strong LD (r^2 = 1.00); and rs1420546 and rs3095611 were in strong LD (r^2 = 0.93).

CHAPTER 7

Genome-wide interaction study of SNPs-by-nitrogen dioxide exposure on respiratory symptoms

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Abstract

Background: Ambient nitrogen dioxide (NO_2) exposure is associated with accelerated loss of lung function and increased prevalence of respiratory symptoms. Whether genetic susceptibility plays a role in the individual response to NO_2 is unclear.

Objective: To identify novel susceptibility loci for air pollution exposure to NO_2 in relation to the development of respiratory symptoms.

Methods: We performed a genome-wide interaction (GWI) study using 10,762 subjects from the LifeLines cohort study. NO_2 exposure at their home address was estimated using Land Use Regression models. All single nucleotide polymorphisms (SNPs) with a SNP-by-NO_2 interaction p-value < 5×10^{-5} were selected and we assessed whether the selected SNPs were cis-acting expression (mRNA) quantitative trait loci in lung tissue.

Results: We found a total of 32 SNPs that interacted with ambient NO_2 exposure and were associated with one of more respiratory symtoms (cough, dyspnea, phlegm and wheeze). Several identified loci harbor plausible candidate genes that might be involved in the development of respiratory symptoms, such as *ROCK2*, *GLS* and *KCTD10*. Fifteen of the 32 GWI-SNPs were cis-acting expression quantitative trait loci (eQTL) associated with gene expression in lung tissue. Rs6759490, the strongest eQTL SNP that significantly interacted with high exposure to NO_2 was associated with gene expression of *ROCK2.*

Conclusion: This GWI study on ambient NO_2 exposure in relation to the presence of respiratory symptoms identified several novel genes. Further research should determine whether the identified genes are true susceptibility loci for NO_2 exposure and whether these SNP-by-exposure interactions consequently contribute to the development of respiratory symptoms.

Introduction

Nitrogen dioxide (NO_2) is an important ambient traffic-related air pollutant which exposure levels are regulated by European and national legislations (1). Ambient NO_2 exposure can cause injury to the lung epithelial cells and may contribute to airway remodeling (2). Exposure to ambient NO_2 has been linked to a wide range of effects on human health, including decreased lung function (3-5), increased respiratory symptoms (5-7), exacerbations of diseases (8,9), hospitalizations (10-12), and mortality (13-15). It is known that exposure to air pollution, such as particles and NO_2, is a risk factor for respiratory symptoms (5,6). However, not all exposed subjects develop respiratory symptoms (6), which suggest that not everybody is equally susceptible to the health effects of air pollution. Susceptibility can be determined by genes and gene-environment interaction studies are important to identify the most susceptible groups.

Genome-wide interaction (GWI) studies are hypothesis-free approaches testing hundreds of thousands gene-by-environment interactions across the whole genome (16-19) on a trait or outcome of interest. For instance, Ierodiakonou et al investigated genetic susceptibility to NO_2 exposure on lung function (FEV_1 and FVC) in both Caucasians and African-American children (16). This study identified and replicated three genome-wide significant SNPs in *MR-1*, *METTL5* and *PTCHD1-AS.* Yet, little is known about the role of genetic factors on respiratory symptoms and if genetic factors modify the effects of NO_2 exposure on respiratory symptoms. GWI studies may provide insights into the role of NO_2 exposure in relation to genetic determinants in the etiology of respiratory symptoms, by exploring potential mechanisms and molecular pathways. In addition, expression quantitative trait loci (eQTLs) are genomic loci that contribute to variation in expression levels of mRNAs, which provide a new approach for connecting genetic variation with and gene expression and may shed light on novel respiratory disease mechanisms (20).

To date, no GWI study has been performed to investigate SNP-by-NO_2 exposure interaction effects on respiratory symptoms. The current GWI study aimed to identify novel genetic variants which interact with NO_2 exposures on respiratory symptoms in a general population cohort. In addition, the function of newly identified SNPs in relation to NO_2 exposure on respiratory symptoms will be analyzed using gene expression analysis in lung tissue.

Methods

Population

We analyzed 10,762 subjects from the LifeLines cohort study (2006-2011). The LifeLines cohort is a large population-based study in the northern part of the Netherlands and was established as a resource for research on complex interactions between environmental, phenotypic and genomic factors in the development of symptoms, diseases and healthy ageing (17,21). The study protocol was approved by the local Medical Ethics Committee of the University Medical Center Groningen, the Netherlands. All participants provided written informed consent.

Genotyping and quality control

Blood samples were genome-wide genotyped using IlluminaCytoSNP-12 arrays. Samples with call rates < 95% were excluded. The quality control criteria of SNPs are listed as follows: SNPs with a genotype call-rate ≥ 95%, minor allele frequency ≥ 1% and Hardy-Weinberg cut-off p-value ≥ 10^{-4} were included. Non-Caucasian samples and first-degree relatives were excluded. A total of 227,981 genotyped SNPs were included in the identification analysis.

Definition of respiratory symptoms

Cough, dyspnea, phlegm and wheeze were defined using the standard questionnaire from the European Community Respiratory Health Survey

(22). Cough was defined as at least one positive answer to the questions: “do you usually cough first thing in the morning in the winter?” or “do you usually cough during the day, or at night, in winter?”; Dyspnea was defined as a positive answer to the question: “are you troubled by shortness of breath when hurrying on level ground or walking up a slight hill or stairs at normal pace?”; Phlegm was defined as at least one positive answer to the questions: “do you usually bring up any phlegm from your chest first thing in the morning in winter?” or “do you usually bring up any phlegm from your chest during the day, or at night, in winter?”; Wheeze was defined as a positive answer to the question: “have you ever suffered from wheezing?” .

Air pollution exposure assessment

Air pollution estimates for the participant’s home address were derived using land use regression (LUR) models developed within the ESCAPE study (23). Estimates of NO_2 levels were calculated using Western European-wide (EU-wide) LUR models enhanced with geographic information system (GIS)-derived land use, road network, and topographic data, as well as satellite-derived estimates of ground-level air pollution. Detailed descriptions of model development and validation can be found in the previous study (24).

Statistical analysis

The associations between NO_2 and respiratory symptoms were investigated using logistic regression adjusted for age, gender and current smoking. NO_2 was investigated as a continuous variable and as dichotomized variable (low and high exposure levels to NO_2 were defined based on the median value (low exposure: < 16.1 μg/m^3; high exposure: ≥ 16.1 μg/m^3)). The effects of SNP-by-NO_2 exposure (standardized per 10 μg/m^3 as continuous variable) interaction effects on the respiratory symptoms cough, dyspnea, phlegm and wheeze were tested using logistic regression adjusted for sex, age, and current smoking using the software package PLINK version 1.07 (25). SNPs were tested in an

additive genetic model. SNPs with a SNP-by-NO_2 interaction p-value < 5×10^{-5} were selected for further analysis. SNP annotation was performed using HaploReg version 4.1 (Broad Institute). The database of GWASCENTRAL (http://www.gwascentral.org/) was used to investigate if the selected SNPs were associated with other respiratory phenotypes such as asthma and forced expiratory volume in 1 second (FEV_1) by previous genome-wide association studies ($p < 0.05$) (26).

Gene expression analysis

We assessed whether the selected SNPs were cis-acting expression (mRNA) quantitative trait loci (cis-eQTLs) in lung tissue which was collected from patients who underwent lung resection surgery at three participating sites: University of Groningen, Laval University, and University of British Columbia (27). Genetic profiles were obtained using Illumina Human1M-Duo BeadChip arrays and gene expression profiles were obtained using a custom Affymetrix array (GEO accession number GPL10379 and GSE23546). A total of 1,087 subjects with no missing data were included in the analysis, and probe sets located within 1 Mb (in both directions) of the selected SNPs were chosen (2 Mb window). Associations between the SNP genotypes and gene expression levels (log2 transformed) were tested by linear regression analysis adjusted for disease status, age, gender, smoking status and cohort specific principal components. Associations were evaluated in each cohort individually and then meta-analyzed. A cis-eQTL was defined as a significant association between a SNP and expression levels of a gene within a 1 Mb (in both directions) distance of that SNP with a p-value below the Bonferroni corrected threshold ($p = 0.05$/number of probe sets within the 2 Mb window) in the meta-analysis.

Results

Population descriptives

Characteristics of the study population and the level of NO_2 exposures are shown in Table 1. A total of 10,762 subjects were studied. The median (min-max) age was 47.8 (18.1-89.9) and median (interquartile range, IQR) for NO_2 was 16.1 (14.0-18.4) $\mu g/m^3$. The prevalence of cough, dyspnea, phlegm and wheeze was 10.1%, 8.4%, 25.7% and 25.6%, respectively (Table 1).

Table 1. Characteristics of the subjects included in the LifeLines cohort.

	Identification
No. with nonmissing data	10,762
Male, n (%)	5,532 (51.4)
Age (yrs), median (min-max)	47.8 (18.1-89.9)
Current smoking, n (%)	2,955 (27.5)
Respiratory symptoms, n (%)	
Cough	1,084 (10.1)
Dyspnea	905 (8.4)
Phlegm	2,770 (25.7)
Wheeze	2,757 (25.6)
Nitrogen dioxide ($\mu g/m^3$), median (IQR)	16.1 (14.0-18.4)

Table 2. Interactions between SNPs (additive effect for minor allele A1) and nitrogen dioxide exposure on cough, dyspnea, phlegm and wheeze with p-value < 5×10^{-5} in the analysis (LifeLines). Minor allele frequency (MAF) is given for A1.

SNP	Chr	A1	MAF	SNP		NO_2		SNP*NO_2		Gene
				OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	
Cough										
rs11208514	1	T	0.49	0.31 (0.19-0.51)	3.23×10^{-6}	0.67 (0.50-0.88)	4.96×10^{-3}	1.95 (1.46-2.61)	5.87×10^{-6}	20kb 3' of *CACHD1*
rs11654001	17	G	0.25	0.33 (0.20-0.56)	2.63×10^{-5}	0.73 (0.56-0.95)	1.75×10^{-2}	1.94 (1.43-2.62)	1.79×10^{-5}	18kb 3' of *FZD2*
rs3784556	15	A	0.19	5.72 (2.52-13.0)	3.02×10^{-5}	1.28 (1.03-1.57)	2.39×10^{-2}	0.34 (0.20-0.56)	2.59×10^{-5}	*PML*
rs11003281	10	A	0.20	3.26 (1.89-5.61)	2.06×10^{-5}	1.45 (1.14-1.85)	2.47×10^{-3}	0.50 (0.36-0.70)	3.78×10^{-5}	185kb 5' of *MBL2*
rs7791529	7	C	0.26	4.07 (2.11-7.85)	2.80×10^{-5}	1.34 (1.07-1.66)	1.04×10^{-2}	0.43 (0.29-0.65)	4.06×10^{-5}	*PRKAG2*
Dyspnea										
rs5996811	22	C	0.27	4.50 (2.40-8.44)	2.76×10^{-6}	1.55 (1.21-1.99)	4.80×10^{-4}	0.40 (0.27-0.59)	3.28×10^{-6}	16kb 5' of *KIAA1671*
rs7984339	13	G	0.44	0.34 (0.21-0.57)	3.00×10^{-5}	0.68 (0.51-0.92)	1.30×10^{-2}	1.97 (1.46-2.66)	8.71×10^{-6}	*TEX26*/*C13orf26*
rs3925038	9	T	0.15	0.32 (0.18-0.58)	1.47×10^{-4}	0.79 (0.61-1.03)	7.80×10^{-2}	2.17 (1.53-3.07)	1.41×10^{-5}	176kb 5' of *RORB*
rs4276378	5	G	0.42	3.20 (1.89-5.42)	1.53×10^{-5}	1.77 (1.32-2.38)	1.31×10^{-4}	0.50 (0.36-0.68)	1.69×10^{-5}	25kb 3' of *MIR4454*
rs11205836	1	T	0.26	0.31 (0.17-0.56)	8.33×10^{-5}	0.79 (0.61-1.03)	8.25×10^{-2}	2.07 (1.47-2.92)	3.33×10^{-5}	*TTC39A*
rs13129382	4	T	0.39	2.75 (1.62-4.66)	1.72×10^{-4}	1.68 (1.27-2.33)	3.02×10^{-4}	0.51 (0.37-0.70)	3.36×10^{-5}	450kb 5' of *NDST3*
rs12647765	16	C	0.22	0.26 (0.12-0.56)	5.18×10^{-4}	0.88 (0.70-1.11)	2.87×10^{-1}	2.52 (1.62-3.92)	4.06×10^{-5}	16kb 5' of *FLJ36777*
rs7188172	5	T	0.10	0.26 (0.14-0.51)	8.68×10^{-5}	0.84 (0.66-1.08)	1.69×10^{-1}	2.29 (1.54-3.40)	4.14×10^{-5}	72kb 3' of *IRX3*

Phlegm										
rs10805145	4	C	0.46	4.10 (2.18-7.74)	1.28×10^{-5}	1.95 (1.27-2.99)	2.23×10^{-3}	0.43 (0.29-0.62)	1.18×10^{-5}	*GABRA2*
rs10850234	12	C	0.15	0.15 (0.07-0.36)	1.39×10^{-5}	0.63 (0.45-0.88)	6.46×10^{-3}	2.95 (1.81-4.81)	1.42×10^{-5}	*KCTD10*/*UBE3B*
rs10075489	5	T	0.27	0.26 (0.13-0.51)	8.32×10^{-5}	0.46 (0.22-0.70)	3.27×10^{-4}	2.44 (1.63-3.66)	1.49×10^{-5}	440kb 3' of *MIR3660*
rs7097666	10	G	0.29	0.25 (0.13-0.48)	2.97×10^{-5}	0.43 (0.27-0.67)	2.20×10^{-4}	2.37 (1.60-3.50)	1.67×10^{-5}	140kb 3' of ANKRD30A
rs12987113	2	A	0.34	4.02 (2.07-7.77)	3.73×10^{-5}	1.72 (1.16-2.56)	7.53×10^{-3}	0.43 (0.29-0.64)	3.00×10^{-5}	*GLS*
rs8180657	6	T	0.38	0.23 (0.12-0.46)	2.26×10^{-5}	0.51 (0.34-0.76)	9.71×10^{-4}	2.34 (1.57-3.50)	3.05×10^{-5}	162kb 5' of *TNFAIP3*
rs2257460	13	T	0.24	0.23 (0.10-0.50)	2.27×10^{-4}	0.59 (0.41-0.84)	3.31×10^{-3}	2.67 (1.68-4.24)	3.20×10^{-5}	*DCUN1D2*
rs3771313	2	T	0.39	0.29 (0.16-0.56)	1.73×10^{-4}	0.41 (0.25-0.67)	3.67×10^{-4}	0.44 (0.29-0.65)	4.62×10^{-5}	*GLS*
rs4953986	2	T	0.21	0.21 (0.11-0.43)	1.57×10^{-5}	0.51 (0.34-0.76)	1.01×10^{-3}	2.39 (1.57-3.63)	4.86×10^{-5}	*NCKAP5*
Wheeze										
rs7427716	3	T	0.09	3.95 (2.05-7.62)	4.20×10^{-5}	1.33 (1.09-1.62)	4.27×10^{-3}	0.49 (0.26-0.58)	4.50×10^{-6}	*CACNA2D3*
rs6759490	2	T	0.21	0.28 (0.16-0.48)	4.08×10^{-6}	0.83 (0.67-1.03)	8.49×10^{-2}	2.07 (1.51-2.86)	8.31×10^{-6}	*ROCK2*
rs4888616	16	T	0.18	2.50 (1.64-3.82)	2.30×10^{-5}	1.68 (1.30-2.19)	9.99×10^{-5}	0.56 (0.43-0.72)	8.50×10^{-6}	2.3kb 3' of *ADAMTS18*
rs1409390	10	C	0.20	0.25 (0.13-0.48)	3.96×10^{-5}	0.87 (0.71-1.06)	1.75×10^{-1}	2.42 (1.64-3.57)	8.55×10^{-6}	*FAM107B*
rs1532983	14	A	0.35	0.42 (0.28-0.64)	3.73×10^{-5}	0.68 (0.52-0.90)	6.97×10^{-3}	0.51 (0.37-0.70)	2.77×10^{-5}	246kb 3' of *LOC100505967*
rs4903526	14	A	0.44	0.43 (0.29-0.65)	4.63×10^{-5}	0.71 (0.54-0.93)	1.26×10^{-2}	1.68 (1.32-2.15)	3.12×10^{-5}	*LOC283575*
rs12032558	1	T	0.23	0.15 (0.06-0.39)	1.08×10^{-4}	0.95 (0.79-1.14)	5.55×10^{-1}	3.18 (1.84-5.51)	3.55×10^{-5}	*TRIM67*
rs1471757	11	A	0.30	2.61 (1.66-4.11)	3.28×10^{-5}	1.47 (1.17-1.85)	9.35×10^{-4}	0.57 (0.43-0.74)	3.93×10^{-5}	*LOC105376567*
rs7232617	18	T	0.18	0.26 (0.14-0.51)	8.03×10^{-5}	0.89 (0.73-1.09)	2.67×10^{-1}	2.24 (1.52-3.31)	4.63×10^{-5}	*DCC*
rs7922216	10	A	0.30	0.32 (0.19-0.55)	2.65×10^{-5}	0.83 (0.67-1.03)	9.62×10^{-2}	1.91 (1.40-2.60)	4.99×10^{-5}	*LOC728558*

NO_2 exposure and respiratory symptoms

There was no significant association between NO_2 as a continuous variable and respiratory symptoms. However, a high level of exposure to NO_2 was significantly associated with respiratory symptoms: cough (odds ratio (OR): 1.09), dyspnea (OR: 1.06), phlegm (OR: 1.10) and wheeze (OR: 1.11) (Table S1).

SNP-by-NO_2 exposures interactions and respiratory symptoms

Detailed information about each model, i.e. the SNP, NO_2 and SNP-by-NO_2 interaction effects on cough, dyspnea, phlegm and wheeze can be found in Table 2. In total, 32 SNPs had a p-value for the interaction term < 5×10^{-5}; 5 for cough, 8 for dyspnea, 9 for phlegm, and 10 for wheeze. There was no overlap in the SNPs between the different symptom-outcomes.

To better understand the SNP-by-NO_2 interaction effects on respiratory symptoms, NO_2 was divided into low and high exposure based on the median value, and we investigated the associations with respiratory symptoms for the different combinations of exposure and genotype using low NO_2 exposure and the wildtype genotype as the reference. For instance, subjects carrying the minor allele of rs6759490 in *ROCK2* have a higher risk for wheezing when they are exposed to high levels of NO_2 compared to subjects exposed to low levels of NO_2 or subjects not carrying the minor allele (Figure 1A). Subjects homozygous for the major allele of rs3771313 in *GLS* have a higher risk for phlegm when they are exposed to high levels of NO_2 compared to subjects exposed to low levels of NO_2 or subjects carrying one or two of the minor alleles (Figure 1C). Subjects carrying the minor allele of rs10850234 in *KCTD10* have a higher risk for phlegm when they are exposed to high levels of NO_2 compared to subjects exposed to low levels of NO_2 or subjects not carrying the minor allele (Figure 1E). These results showed that individuals carrying the risk allele at specific genetic loci are more susceptible to the effects of NO_2 on respiratory symptoms.

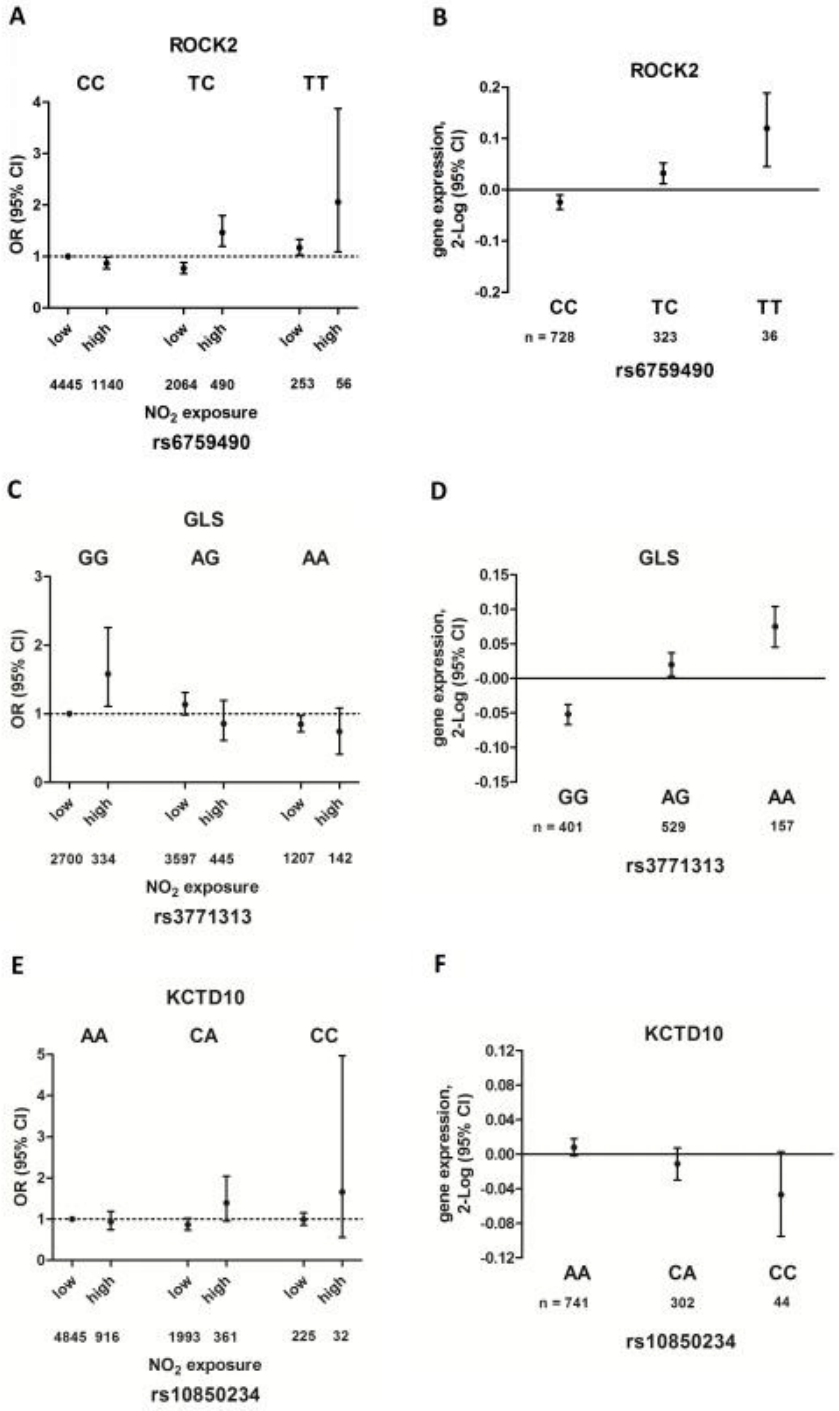

Figure 1. A: Associations between the SNP rs6759490 and wheeze in subjects with low and high exposure to NO_2 (based on the median value (low exposure: < 16.1 $\mu g/m^3$; high exposure: ≥ 16.1 $\mu g/m^3$)). **B:** Gene expression levels in lung tissue stratified by genotype for SNP rs6759490. **C:** Associations between the SNP rs3771313 and phlegm in subjects with low and high exposure to NO_2. **D:** Gene expression levels in lung tissue stratified by genotype for SNP rs3771313. **E:** Associations between the SNP rs10850234 and phlegm in subjects with low and high exposure to NO_2. **F:** Gene expression levels in lung tissue stratified by genotype for SNP rs10850234.

Association in previous GWA studies

According to the GWAS CENTRAL database, rs3784556 located in *PML*, rs10850234 located in *KCTD10*, rs12987113 and rs3771313 located in *GLS*, rs4953986 located in *NCKAP5*, rs7427716 located in *CACNA2D3*, and rs6759490 located in *ROCK2* have been associated with lung function, asthma or IgE levels with a $p< 0.05$ in previous GWA studies (Table S2).

Expression Quantitative Trait Loci (eQTL)

We investigated whether the 32 identified SNPs were associated with gene expression levels in lung tissue from 1,087 patients. Six SNPs were not available in the eQTL-database and a proxy-SNP in high LD was used. A total of 15 SNPs showed cis-eQTL associations with p-values below the Bonferroni corrected threshold (Table 3, Figures 1B, 1D, 1F). In Table S3 the total number of tested probe sets per SNP and the total number of significantly associated probe sets are given. The strongest cis-eQTL association was found for SNP rs6759490 associated with expression of *ROCK2*.

Table 3. Expression Quantitative Trait Loci (eQTL). Results of the gene expression analysis in lung tissue for the identified SNPs.

SNP	Chr	Effect allele	Probe set	B	SE	P-value	Threshold	Gene
Cough								
rs11654001	17	G	100138748_TGI_at	-0.12	0.02	1.69×10^{-14}	4.90×10^{-4}	*DBF4B*
			100159869_TGI_at	-0.10	0.01	4.86×10^{-12}		*DBF4B*
			100126590_TGI_at	-0.10	0.02	3.84×10^{-10}		*HIGD1B*
			100131975_TGI_at	0.10	0.02	2.04×10^{-9}		*FZD2*
			100134748_TGI_at	-0.06	0.01	1.44×10^{-5}		*MEIOC*
			100137357_TGI_at	0.04	0.01	1.48×10^{-4}		*CCDC103*
rs7791529	7	C	100307093_TGI_at	-0.06	0.02	2.73×10^{-5}	6.49×10^{-4}	*PRKAG2*
			100139041_TGI_at	-0.04	0.01	4.78×10^{-4}		*PRKAG2*
Dyspnea								
rs11205836	1	T	100140992_TGI_at	0.04	0.01	1.22×10^{-13}	1.06×10^{-3}	*EPS15*
			100155271_TGI_at	-0.03	0.01	1.29×10^{-5}		*EPS15*
			100307731_TGI_at	0.03	0.01	6.76×10^{-5}		*EPS15*
rs12647765	16	C	100152186_TGI_at	0.06	0.01	2.86×10^{-6}	9.80×10^{-4}	*GRPEL1*
rs13129382	4	T	100305239_TGI_at	0.05	0.02	1.68×10^{-3}	5.56×10^{-3}	*NT5C3AP1*
Phlegm								
rs10850234	12	C	100141932_TGI_at	-0.16	0.02	2.99×10^{-19}	5.88×10^{-4}	*MMAB*
			100142774_TGI_at	0.08	0.01	5.63×10^{-15}		*MMAB*
			100160282_TGI_at	0.14	0.02	2.94×10^{-13}		*MYO1H*

			100303074_TGI_at	0.14	0.02	1.60×10^{-12}		*MYO1H*
			100157772_TGI_at	-0.05	0.01	1.22×10^{-7}		*MMAB*
			100313700_TGI_at	0.05	0.01	1.03×10^{-6}		*UBE3B*
			100131129_TGI_at	-0.07	0.02	5.22×10^{-5}		*ACACB*
			100142416_TGI_at	0.03	0.01	1.04×10^{-4}		*UBE3B*
			100129614_TGI_at	0.03	0.01	1.14×10^{-4}		*MVK*
			100154146_TGI_at	-0.03	0.01	2.64×10^{-4}		*KCTD10*
rs6758866	2	A	100122304_TGI_at	0.06	0.01	1.58×10^{-15}	1.16×10^{-3}	*GLS*
			100310147_TGI_at	-0.04	0.01	1.26×10^{-4}		*HIBCH*
			100161306_TGI_at	0.04	0.01	1.74×10^{-4}		*GLS*
rs3771313	2	T	100122304_TGI_at	0.06	0.01	2.92×10^{-15}	1.16×10^{-3}	*GLS*
			100310147_TGI_at	-0.04	0.01	1.28×10^{-4}		*HIBCH*
			100161306_TGI_at	0.04	0.01	1.66×10^{-4}		*GLS*
rs7097666	10	G	100304014_TGI_at	0.09	0.02	9.97×10^{-9}	1.79×10^{-3}	*ZNF25*
			100124863_TGI_at	-0.04	0.01	1.00×10^{-5}		*ZNF33A*
			100154037_TGI_at	0.08	0.02	5.37×10^{-5}		*ZNF25*
			100127547_TGI_at	0.04	0.01	1.97×10^{-4}		*ZNF37A*
			100304941_TGI_at	-0.04	0.01	3.77×10^{-4}		*ZNF33A*
			100131654_TGI_at	0.03	0.01	9.16×10^{-4}		*ZNF248*
			100302826_TGI_at	0.03	0.01	9.61×10^{-4}		*ZNF37A*
rs4953986	2	T	100145759_TGI_at	0.07	0.01	5.42×10^{-7}	2.94×10^{-3}	*NCKAP5*
rs10805145	4	C	100152195_TGI_at	0.03	0.01	9.95×10^{-4}	1.16×10^{-3}	*GABRA2*
			100308388_TGI_at	0.04	0.01	1.48×10^{-3}		*GABRA2*

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Wheeze								
rs6759490	2	T	100311377_TGI_at	-0.08	0.01	2.66×10^{-22}	1.35×10^{-3}	*ROCK2*
			100159613_TGI_at	0.08	0.01	1.59×10^{-14}		*ROCK2*
			100306938_TGI_at	0.06	0.01	9.37×10^{-10}		*ROCK2*
			100133766_TGI_at	0.08	0.01	1.06×10^{-9}		*ROCK2*
			100160466_TGI_at	-0.07	0.01	2.58×10^{-8}		*ROCK2*
			100124316_TGI_at	-0.03	0.01	2.38×10^{-4}		*PQLC3*
rs2162957	16	G	100122211_TGI_at	0.05	0.01	4.59×10^{-14}	3.13×10^{-3}	*MON1B*
			100126943_TGI_at	-0.11	0.02	2.93×10^{-6}		*ADAMTS18*
rs4903526	14	A	100154109_TGI_at	-0.03	0.01	1.53×10^{-5}	1.16×10^{-3}	*C14orf4*
rs1532983	14	A	100154109_TGI_at	-0.03	0.01	1.19×10^{-4}	1.16×10^{-3}	*C14orf4*

SE = standard error, threshold was calculated as p value = 0.05/number probe sets within the 2 Mb window. Rs6758866 and rs12987113 were in strong LD ($r^2 = 0.98$); and rs2162957 and rs4888616 were in strong LD ($r^2 = 0.92$).

Discussion

This is the first hypothesis-free genome-wide SNP-by-NO_2 exposure interaction study on respiratory symptoms including cough, dyspnea, phlegm and wheeze. We found that exposure to high ambient NO_2 (≥ 16.1 μg/m^3) at the participant's home address was associated with a higher prevalence of respiratory symptoms. We identified 32 SNP-by-NO_2 interactions associated with the presence of symptoms. In previous GWA studies, 7 of these SNPs were associated with lung function levels, asthma, or IgE levels. According to the eQTL analysis in lung tissue, 15 of these SNPs were significantly associated with mRNA expression levels of nearby genes. In a word, this GWI study demonstrated that SNP-by-NO_2 exposure interaction effects may play an important role in respiratory symptoms.

The functional meaning of the identified SNPs interacting with NO_2 exposure on respiratory symptoms was explored by investigating the level of gene expression in lung tissue using cis-eQTLs analysis. In the cis-eQTL analysis, we identified 15 SNPs that were associated with expression of nearby genes in lung tissue. The strongest cis-eQTL association was found for rs6759490 associated with expression of *ROCK2*. Cloutier et al showed that *ROCK2* is a key regulator in tracheal occlusion (TO)-induced accelerated lung development (28). Zanin-Zhorov et al demonstrated that *ROCK2* regulate the secretion of proinflammatory cytokines, such as IL-17 and IL-21 and the development of autoimmunity (29). In addition, *ROCK2* plays an essential role in the maintenance of geometric shape and polarization of epithelial cells which in lungs provide structural integrity, moisten and protect the airways (30). In the current study, exposure to NO_2 was associated with a higher risk of wheezing only in subjects carrying the minor allele for rs6759490 indicating the potential importance of *ROCK2* in determining the individual susceptibility to the harmful effects of NO_2.

The second interesting SNP is rs3771313, which shows a higher risk of phlegm in association with NO_2 exposure only in subjects having the two major G-alleles of this SNP. In addition, these GG-subjects have a lower expression of the gene *GLS*. *GLS* encodes the mitochondrial glutaminase which catalyzes the hydrolysis of glutamine to glutamate and ammonia. Hunt et al showed that glutaminase expression and ammonia levels are diminished in asthmatic lung and expired breath condensate, respectively (31). Glutaminase protein was expressed in the human airway epithelium and may be relevant both to the regulation of airway pH and to the pathophysiology of acute airway inflammation (31-33). Overall, our results suggest that lower expression of glutaminase increases the susceptibility to NO_2 exposure and may result in the development of respiratory symptoms.

The third SNP of interest is rs10850234, located on *KCTD10*. *KCTD10*, a member of the polymerase delta-interacting protein 1 (PDIP1) gene family, participates in DNA repair, DNA replication and cell-cycle control (34). *KCTD10* is highly expressed in lung and heart, and may play an important role in tissue development (35-37). Ren *et al.* reported that *KCTD10* is involved in embryonic angiogenesis and heart development by negatively regulating the Notch signaling pathway (33). In our study subjects carrying the minor allele of rs10850234 had lower expression of *KCTD10* and a higher risk for phlegm in association with NO_2 exposure. This might suggest that lower expression of *KCTD10* may increase the susceptibility to develop respiratory symptoms in relation to NO_2 exposure.

The strengths of the current study, in which we investigated SNP-by-NO_2 exposures interaction effects on respiratory symptoms in the LifeLines cohort, are a large number of subjects and the associations with the gene expression level of the identified SNPs in lung tissue. A limitation of the study is the low NO_2-level and the small variation in NO_2-level between the subjects. However, even with these low NO_2-exposure levels we find

significant gene-by-NO_2 exposure interactions in plausible genes. Another limitation of this study is the lack of replication, so we will look for a replication cohort to confirm our findings in this study.

In conclusion, this is the first genome-wide gene-environment interaction study that investigated interactions of SNPs with NO_2 exposure on respiratory symptoms cough, dyspnea, phlegm, and wheeze. We identified several plausible candidate genes that may be involved in biological pathways resulting in lung function impairment and respiratory symptoms, i.e. *ROCK2, GLS* and *KCTD10*. Further research should find another independent study to replicate our findings, and determine whether the identified genes are true susceptibility loci for respiratory symptoms. This information may contribute to the understanding of pathways underlying the development of respiratory symptoms and to the identification of subjects that are genetically susceptible to environmental exposures.

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Table S1. Association between NO_2 and respiratory symptoms using multiple logistic regression analyses.

NO_2	Cough OR (95% CI)	Dyspnea OR (95% CI)	Phlegm OR (95% CI)	Wheeze OR (95% CI)
Continuous	1.006 (0.986-1.026)	1.010 (0.989-1.031)	1.006 (0.983-1.029)	1.007 (0.989-1.025)
Dichotomous[a]	1.090 (1.042-1.141)***	1.059 (1.009-1.111)***	1.102 (1.046-1.161)***	1.113 (1.069-1.158)***

Abbreviations, NO_2: nitrogen dioxide; OR: odds ratio; CI: confidence interval; logistic regression models adjusted for age, gender and current smoking. [a] NO_2 was divided into low and high exposure based on the median value (low exposure: < 16.1 μg/m^3; high exposure: ≥ 16.1 μg/m^3); ***: $p < 0.001$.

Table S2. Associations between the SNPs and phenotype according to the GWAS Central Database.

SNP	Gene	Phenotype	P-value
rs3784556	*PML*	Asthma	0.02
rs10850234	*KCTD10*	Asthma	0.03
		Serum total IgE concentration	0.03
rs12987113	*GLS*	Pulmonary function (FEV_1)	0.01
rs3771313	*GLS*	Pulmonary function (FEV_1)	0.009
rs4953986	*NCKAP5*	Pulmonary function (FEV_1)	0.04
rs7427716	*CACNA2D3*	Pulmonary function (FEV_1/FVC)	0.03
		Pulmonary function (FEV_1)	0.006
rs6759490	*ROCK2*	Pulmonary function (FEV_1/FVC)	0.005
		Pulmonary function (FEV_1)	0.04

Information was obtained from GWASCENTRAL database (http://www.gwascentral.org/); IgE: immunoglobulin E; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity.

Table S3. SNPs acting as lung cis-eQTLs on genes within a 1 Mb distance.

SNP	Number of probe sets in window	Number of probe sets significant
Cough		
rs11208514	0	0
rs11654001	102	6
rs3784556	71	0
rs11003281/ rs2840323	11	0
rs7791529	77	2
Dyspnea		
rs5996811/ rs2064136	49	0
rs7984339	25	0
rs3925038/ rs4324976	18	0
rs4276378	28	0
rs11205836	47	3
rs13129382	9	1
rs12647765	51	1
rs7188172/ rs2665278	26	0
Phlegm		
rs10805145	14	2
rs10850234	85	10
rs10075489	0	0
rs7097666	28	7
rs12987113/ rs6758866	43	3
rs8180657	35	0

rs2257460	0	0
rs3771313	43	3
rs4953986	17	1
Wheeze		
rs7427716	31	0
rs6759490	37	6
rs4888616/ rs2162957	16	2
rs1409390	46	0
rs1532983	43	1
rs4903526	43	1
rs12032558	43	0
rs1471757	25	0
rs7232617	15	0
rs7922216	0	0

Rs2840323 and rs11003283 were in strong LD (r^2 = 0.99); rs2064136 and rs5996811 were in strong LD (r^2 = 0.98); rs4324976 and rs3925038 were in strong LD (r^2 = 0.95); rs2665278 and rs7188172 were in strong LD (r^2 = 0.87); rs6758866 and rs12987113 were in strong LD (r^2 = 0.98); and rs2162957 and rs4888616 were in strong LD (r^2 = 0.92).

CHAPTER 8

Summary, discussion and future perspectives

Summary

The aim of this PhD project was to assess whether environmental exposures, such as electronic waste (e-waste) exposure, occupational exposure and air pollution exposure, are associated with the level of lung function and the prevalence of respiratory symptoms. In addition, we also were interested in evaluating differences in genetic susceptibility to these exposures between individuals. The studies described in this thesis have shown that environmental exposures are associated with lower lung function levels and a higher prevalence of respiratory symptoms. We identified several genetic polymorphisms that are associated with individual susceptibility to occupational and nitrogen dioxide exposure.

Chapter 1 introduces the study. The environmental risk factors of interest are explained and more information about the used outcome measures is given. In addition, the methods of studying genetic associations are described. Finally, the study populations used in the thesis are introduced.

Chapter 2 reviews studies investigating the adverse effects of e-waste on the human body. We assessed cross-sectional associations between e-waste exposures to toxic heavy metals and organic pollutants and impaired human health including endocrine toxicity, respiratory toxicity, developmental toxicity, reproductive toxicity, neurotoxicity, and genetic toxicity. In this study, we concluded that heavy metals and organic pollutants derived from e-waste can accumulate in environmental media such as air, soil, dust, water, and in the human body directly by inhalation, ingestion and dermal absorption, or indirectly by the food chain. Exposure to e-waste has adverse effects on human health such as endocrine disturbances, decline in lung function, developmental delay, sperm damage, mental decline, and DNA and protein damage.

Chapter 3 describes a review in which we assessed the adverse effects of a number of heavy metal pollutants from an e-waste recycling area on the health of children, including respiratory, cardiovascular, nervous, immune, reproductive, skeletal, and urinary health. Children can become exposed to heavy metals in air, dust, soil, sediment, water, and food sources through several routes that include ingestion, inhalation, and dermal absorption from combustion, discharges and manufacturing facilities. Children are considered more susceptible to hazardous metal substances compared to adults for several reasons such as excess routes of exposure, a higher basal metabolic rate, and a lower toxin elimination rate. We found that the concentration of heavy metals in the environmental media such as air, dust, soil, sediment, and water is higher in an e-waste exposed area than in the reference areas. Living in e-waste exposed areas is associated with higher heavy metal concentrations in children's blood and heavy metal exposure is associated with body system impairment.

Chapter 4 describes a study in which we assessed the association between living in an e-waste exposed area and levels of heavy metals in the blood in pre-school children. In addition, we investigated the association between blood heavy metals levels and the level of lung function, and the association between living in an e-waste exposed area and level of lung function in these children. We conclude that living in e-waste exposed areas is associated with higher levels of blood lead, platelets and thrombocytocrit, and lower levels of hemoglobin, hematocrit and lung function. In addition, hemoglobin was associated with higher lung function levels.

Chapter 5 describes a number of genome-wide association studies in which we aimed to identify and replicate novel genetic loci that associated with respiratory symptoms including cough, dyspnea and phlegm. We performed an identification analysis in 7,976 subjects from the first data release of the LifeLines cohort study (LifeLines I), and replicated our initial

findings in 5,260 subjects from the second data release of the LifeLines cohort study (LifeLines II) and in 1,529 subjects from the Vlagtwedde-Vlaardingen cohort. We identified and replicated one SNP rs16918212, located on *A2MP1* associated with cough. However, the gene *A2MP1*, a pseudogene, has not been associated with lung function impairment or respiratory diseases in literature. Therefore, this gene might not be a plausible gene underlying the development of respiratory symptoms.

Chapter 6 describes a genome-wide interaction study in which we aimed to identify novel susceptibility loci that affect individual susceptibility to common occupational exposures, i.e. vapors, gas, dust or fumes (VGDF), biological dust, mineral dust, gas and fumes, pesticides, herbicides, insecticides, aromatic solvents, chlorinated solvents, other solvents, and metals, on respiratory symptoms including cough, dyspnea and phlegm. First, we performed an identification analysis in 7,976 subjects from the first data release of the LifeLines cohort study (LifeLines I), then verified our initial findings in 5,260 subjects from the second data release of the LifeLines cohort study (LifeLines II) and in 1,529 subjects from the Vlagtwedde-Vlaardingen cohort using meta-analysis of the interaction effects. Additionally, we assessed whether these SNPs were cis-eQTLs in lung tissue. We identified and replicated 11 SNPs that interacted with one of the occupational exposures. Several identified SNPs were located in genes that may be associated with respiratory health, i.e. *TMPRSS9, TOX3* and *ARHGAP18*. Of these 11 replicated SNPs, 3 SNPs were cis-acting eQTLs associated with gene expression of their neighboring genes *FCER1A, CHN1* and *TIMM13*. Further research should assess whether the identified genes are true genetic loci for the development of respiratory symptoms.

Chapter 7 describes a genome-wide interaction study in which we aimed to identify novel susceptibility loci that affect individual susceptibility to NO_2 exposure on respiratory symptoms including cough, dyspnea,

phlegm and wheeze. SNP by NO_2 exposure interactions were assessed in 10,762 subjects from the LifeLines cohort study. Furthermore, we assessed whether these SNPs that interacted with NO_2 on respiratory symptoms were cis-eQTLs in lung tissue. Several identified loci were plausible genes associated with lung function levels in the literature, i.e. *ROCK2*, *GLS* and *KCTD10*. Of the 32 identified SNPs, 15 SNPs were cis-eQTLs associated with gene expression of their nearby genes such as *DBF4B, PRKAG2, EPS15, GRPEL1*, *MMAB*, *GLS*, *ZNF25*, *NCKAP5*, *GABRA2*, *ROCK2*, *MON1B* and *C14orf4* in lung tissue. The most interesting SNPs was located in the gene *ROCK2.* We found that subjects carrying the minor allele of the SNP had a significantly higher *ROCK2* gene expression in lung tissue and also had a greater risk to wheeze in association with NO_2 exposure, compared to subjects carrying the 2 major alleles of the SNP. This data provides additional support to explore a possible role of these genes in the development of respiratory symptoms. Further studies should replicate these results in other independent cohorts, and determine whether the identified genes are true susceptibility loci for respiratory symptoms due to exposure to ambient NO_2, and whether these SNP-by-NO_2 exposure interactions consequently increase the risk to develop lung diseases such as asthma and COPD.

Discussion

Environmental exposure and respiratory symptoms and levels of lung function

E-waste exposures and respiratory symptoms and levels of lung function

Electronic waste (e-waste), from electrical and electronic equipment or products after reaching the end of their useful life, has become the largest growing amount of waste in the world (1, 2). It was estimated that global e-waste generation was 41.8 million tons in 2014 and may increase to 65.4 million tons by 2017 (3). E-waste contains heavy metals and plastics that can be released to the environment during inappropriate recycling

processes and disposal, which could be harmful for humans, animals, vegetation or other environmental materials (4-6). Our recent study showed that levels of lead and cadmium both in $PM_{2.5}$ and blood were higher from the e-waste exposed area than the reference area, and prevalence of respiratory symptoms including cough, dyspnea, phlegm and wheeze was higher in the e-waste exposed area than the reference area (7). Living in e-waste exposed areas was associated with higher heavy metal concentrations in blood including lead and cadmium, and a higher prevalence of respiratory symptoms including cough, dyspnea, phlegm and wheeze in preschool children (7). One of our previous studies showed that the blood levels of three transition metals, i.e. chromium, manganese, and nickel, were higher in children living in the e-waste exposed area than the reference area, but there was no difference in forced vital capacity (FVC). However, in the youngest age group (8-9 years) FVC was significantly lower in children from the e-waste exposed area compared to the reference area (8). This indicates that young children may be sensitive and vulnerable to e-waste exposure because of additional routes of exposure, high-risk behaviors, and their changing physiology compared to older children and adults (1). Therefore, in chapter 4 we performed another study measuring concentrations of lead and cadmium in blood, and lung function levels (FVC and forced expiratory volume in 1 second (FEV_1)) in very young (i.e. preschool) children from the e-waste exposed area and two reference areas. We found that preschool children from the e-waste exposed area have higher concentrations of lead in their blood and lower lung function levels when compared to the reference areas. Furthermore, we assess whether there were associations between concentrations of lead and cadmium in blood, and lung function levels in preschool children, and we found that there was no significant association between them. However, living in the e-waste exposed area was associated with higher lead levels in blood and lower lung function levels in preschool children, which may confirm our previous hypothesis that young children are more susceptible to develop lung function impairment when exposed to e-waste compared to older children.

Occupational exposures and respiratory symptoms and lung function levels

In the studies presented in this thesis, occupational exposures have been divided into four categories: Vapors, gas, dust or fumes (VGDF), including the subcategories biological dust, mineral dust, and gases and fumes; Pesticides, including the subcategories herbicides and insecticides; Solvents, including the subcategories aromatic solvents, chlorinated solvents and other solvents; and Heavy metals such as lead, cadmium, chromium, mercury and nickel (Table 1). Previous studies have shown that VGDF exposure is clearly associated with lower levels of FEV_1 and FEV_1/FVC, and a higher prevalence of large airway obstruction in a sample from the general population (9-11). Small airways are affected by occupational exposure to VGDF, and the subcategories biological dust, gases and fumes as well (12). It is worth to mention that these associations also exist in subjects with normal FEV_1/FVC and FEV_1% predicted values, demonstrating that the effects of VGDF exposure on the small airways are independent of their effects on the large airways (13). Occupational dust exposure is associated with respiratory symptoms including chronic cough, phlegm, and wheeze in general population studies conducted in Europe (13-15), China (16, 17), and USA (18, 19). Specifically exposure to biological dust is associated with a higher risk of emphysema and COPD in the general population (20). Mineral dust exposure is associated with a lower level of FEV_1 and a higher COPD prevalence. Occupational exposure to gases and fumes is associated with airflow obstruction, respiratory symptoms including chronic cough, phlegm and persistent wheeze, and a lower FEV_1/FVC and a lower FEV_1% predicted values compared with subjects without occupational exposure in both men and women (21).

Table 1. Most prevalent occupations in subjects exposed in the LifeLines sample (table modified from De Jong (9,22,44)).

Exposure	Jobs
VGDF	
Biological dust	Dairy and livestock producers, Carpenters, Freight handlers, Bakers and pastry-cooks, Crop and animal producers (mixed), Veterinary assistants, floor layers and tile setters, motorize farm and forestry plant operators
Mineral dust	Welders and flame-cutters, Agricultural and industrial mechanics and fitters, Freight handlers, Gardeners, horticultural and nursery growers, Building and construction laborers, Earth moving and related plant operators, Field crop and vegetable growers, Crop and animal producers (mixed), Construction and maintenance laborers (roads), and Earth moving and related plant operators
Gases and fumes	Heavy truck and lorry drivers, Motor vehicle mechanics, Welders and flame-cutters, Agricultural and industrial mechanics and fitters, Plumbers and pipe fitters, Painters, Butchers, fishmongers and related preparers, and Earth moving and related plant operators
Pesticides	
Herbicides	Field crop and vegetable growers, Gardeners, Horticultural and nursery growers, and Tree and shrub crop growers
Insecticides	Field crop and vegetable growers, Gardeners, Horticultural and nursery growers, Tree and shrub crop growers, and Mixed crop and animal farmers
Solvents	
Aromatic solvents	Painters, Printing machine operators, Varneshers and related painters,
Chlorinated solvents	Motor vehicle mechanics, Agricultural and industrial mechanics sheet metal workers
Heavy metals	Welders and flame-cutters, Motor vehicle mechanics, Agricultural and industrial mechanics, Sheet metal workers

Our previous study showed that occupational pesticide exposure was associated with a lower level of lung function (FEV_1 and FEV_1/FVC) and accelerated decline of lung function (FEV_1 and FEV_1/FVC) consistent with airway obstruction as measured by spirometry in two general population cohorts (22). These associations with pesticides were seen in both men and women and smokers and non-smokers. A series of studies conducted within the Agricultural Health Study, North Carolina showed a

higher prevalence of wheeze, chronic bronchitis and asthma both in the pesticide applicators (farmers) (23-26) as well as in their spouses (27-29). A few small scale cross-sectional studies found associations between specific types of pesticides and lower levels of FEV_1 and FVC in occupationally exposed farmers from Sri-Lanka (30), South Korea (31), Costa Rica (32) and Spain (33). Globally, over 5 billion pounds of pesticides were used in 2006 (34), and more than 1.1 billion workers (approximately 34% of the global working force) were exposed to pesticides in the agricultural sector (35). People living in agriculture-intensive regions may be at risk of pesticide drift exposure from neighboring farms (36). The Netherlands is famous for its high-density population and high-yield agricultural production areas where large numbers of people live near greenhouses and pesticide-treated farmland, and may be at risk of pesticide-related conditions or illnesses such as a lower lung function and a higher prevalence of respiratory symptoms or diseases. To date, knowledge on the association between occupational pesticide exposure and respiratory symptoms is still limited and not fully understood.

Most occupational solvents are complex substances containing a large number of individual specific molecules. For instance, the solvents referred to as “aromatic” are composed primarily of aromatic constituents containing one or more benzene rings. Respiratory and other health effects of solvent exposure depend on the actual inflicted exposure dose. Higher levels of solvent exposure tend to lead to a higher risk of suffering from respiratory symptoms (37). According to the Agricultural Health Study, occupational exposure to solvents from painting and cleaning were associated with higher odds of wheeze in a duration-dependent fashion (38). A cross-sectional study, conducted in gun factory workers, investigated the association between exposure to solvents and asthma-related symptoms including chronic cough, chronic bronchitis and dyspnea. Asthma-related symptoms were more prevalent in exposed workers than unexposed, both in non-smokers and in smokers (39). A systematic review investigating the effects of occupational exposure to formaldehyde on respiratory symptoms and lung function, which showed

that occupational exposure to formaldehyde was associated with a higher prevalence of cough, dyspnea and wheezing in most of the included studies (40). Postnatal exposure to solvent-containing products in the home was associated with a higher risk of wheezing, which remained after adjustment for prenatal exposure (41). Exposure to aromatic solvents, chlorinated solvents and any solvents were associated with the risk of small for gestational age (fetal growth restriction) in a population-based sample of women in the National Birth Defects Prevention Study (42). Preterm babies are likely to have a lower birth weight compared to babies with normal birth conditions, and a low birth weight in children born at term was associated with a higher risk of respiratory symptoms (43). Interesting, occupational exposure to chlorinated solvents was significantly contributing to chronic mucus hypersecretion (CMH) in subjects without COPD, but not at all in subjects with COPD (44).

Occupational exposure to metals is not limited to mining and smelting industries. Metals are also needed for the industrial development and occupational exposure to metals can occur in a diverse spectrum of jobs involved in manufacturing, processing, and application of metals and metal-containing products (45). Previous cohort studies from Norway found that exposure to heavy metals was associated with lower lung function levels, increased lung function loss, and higher prevalence of respiratory symptoms in the same worker population (46-48). Exposure to metalworking fluids was associated with a higher risk of cough, dyspnea, wheezing, chronic bronchitis, and asthma in occupationally exposed workers from southern Finland (49). Environmental heavy metals not only cannot be degraded in the environment, but also can be enrich in humans through ingestion, inhalation, dermal absorption, and food chain. Previous cross-sectional studies conducted in China demonstrated that heavy metal levels such as lead and cadmium in environmental media including air (7, 50), soil (51), dust (52) and water (53) and in blood of children (7) were higher for the e-waste recycling area compared to the reference area. We found that blood chromium was associated with a higher prevalence of cough, and blood manganese was associated with a

higher prevalence of wheeze (7). In addition, other cross-sectional studies showed blood lead and cadmium levels were associated with a lower lung function in adults (54, 55). Finally, in a large general population-based study, CMH was associated with occupational heavy metal exposure in subjects without COPD, but not in subjects with COPD (44).

Nitrogen dioxide (NO_2) is one of the major gaseous air pollutants that continue to raise concern. Traffic pollution is the main outdoor source of NO_2 (56). The most important indoor sources of NO_2 include tobacco smoke and gas-, wood-, oil-, kerosene- and coal-burning appliances such as stoves, ovens, gas and water heaters and fireplaces, and particularly poorly maintained appliances. Outdoor NO_2 from natural and anthropogenic sources and air exchange rates between outdoor and indoor can also influence indoor levels (57). Ambient concentrations of NO_2 vary widely according to local sources. Several studies reported that ambient NO_2 exposure has been associated with lower lung function (58-60), respiratory symptoms (61-63), hospital admissions and mortality (64-66). In a German birth cohort, a higher annual average NO_2 concentration was associated with more bronchial symptoms of children in the first year of life (67). A review showed that long-term NO_2 exposure (below 40 μg NO_2/m^3 World Health Organization limit) was associated with adverse effects including respiratory symptoms/diseases (59). Outdoor NO_2 exposure was Vgests that girls may be more susceptible to indoor NO_2 than boys (62). However, the associations were not consistent in all studies. For instance, the large PEACE study found no consistent effects of outdoor NO_2 on respiratory symptoms or lung function (68). There has been increasing interest both in outdoor NO_2 pollution and in indoor NO_2 pollution in the recent decades, and studies showed that the outdoor NO_2 exposure was higher than indoor NO_2 exposure in the same area (69-72). One of our previous studies showed that higher long-term NO_2 concentrations were associated with lower post bronchodilator FEV_1 and FVC % predicted (73).

Genetic risk factors and respiratory symptoms and levels of lung

function

GWA Studies

A GWA study is a hypothesis-free approach to identify genetic variations associated with a trait or a disease of interest (74). Compared to candidate gene studies focusing on specific known genes, the advantage of GWA studies is that there is no pre-selection of genes and thus novel susceptibility loci associated with a disease or trait can be identified across the entire genome. Although GWA studies were successful in identifying new loci associated with lung function and lung diseases such as asthma and COPD, the newly identified genetic variants have so far only explained a small proportion of the genetic contribution to these complex diseases or traits, and largely disregard environmental factors that may trigger the development of these diseases and traits (75). As we know, respiratory symptoms are associated with accelerated lung function decline (76-78), lung diseases (79-81) and mortality (82-84). To date, only one GWA study on respiratory symptoms (i.e. CMH) has been published (85). Our GWA study (chapter 5) did not identify SNPs that were genome wide significantly ($p < 2.06\times10^{-7}$) associated with the risk of cough, dyspnea and phlegm but we did identify 17, 19 and 14 SNPs associated with cough, dyspnea and phlegm respectively, at a p-value < 10^{-4}. However, these SNPs did not show a convincing association with the presence of respiratory symptoms in the meta-analysis of two independent replication cohorts. This lack of replication can be explained by the fact that respiratory symptoms can be caused by different environmental exposures or can be a presentation of different underlying diseases with specific genetic or environmental origins. Susceptibility to environmental exposures may be genetically determined and susceptibility loci may differ between exposures. The environment should thus be taken into account when studying the association between genetics and respiratory symptoms. Therefore, the next logical step is to perform a genome-wide interaction (GWI) study to identify genetic loci for respiratory symptoms in the interaction with known harmful environmental exposures.

GWI Studies

To date, few studies have comprehensively examined combined genetic and environmental effects on lung function (75, 86, 87), and no study has comprehensively examined the combined genetic and environmental effects on respiratory symptoms. A GWI study is a hypothesis free approach that is used to identify genetic loci that affect the susceptibility for the effects of known harmful exposures on a trait or a disease. Compared to a GWA study identifying genetic variations predisposing for a trait or a disease, a GWIs study is aiming to identify genetic loci based on gene-environmental exposure interactions on a trait or a disease across the entire genome (86). Hancock et al (2012) reported one of the first genome-wide gene-environment interaction studies. In this study the environmental interacting factor (smoking) and the study identified novel loci associated with the level of lung function (FEV_1, FEV_1/FVC) that would have been missed when only focusing on direct genetic effects (87). It is likely that genetic susceptibility is also of importance for occupational exposures and air pollution such as ambient NO_2 exposure. So far, there was no GWI study identifying genetic loci that affect the susceptibility for the effects of environmental exposures such as occupational exposure and ambient NO_2 exposures on respiratory symptoms.

Therefore, in this PhD project we aimed to assess the inter-individual difference in susceptibility for respiratory symptoms including cough, dyspnea, phlegm and wheeze when exposed to occupational exposures and ambient NO_2. With the studies presented in this thesis we are the first assessing gene-environment interaction effects on respiratory symptoms in a genome-wide hypothesis-free manner. The results show that the identified genetic variants based on gene-environmental exposure interactions are associated with respiratory symptoms; therefore they are plausible candidate genes. In order to obtain additional insight in the biological plausibility of these genes and potential pathways engaged in the development of respiratory symptoms, we have performed gene expression analysis. In chapter 6 we have performed a gene expression analysis and found that 4 of the SNPs in interaction with biological dust and mineral dust exposure were cis-eQTLs in lung tissue. In chapter 7 we

performed a GWI analysis of gene by ambient NO_2 exposure interaction, and found that 14 of the SNPs in interaction with ambient NO_2 exposure exposures were cis-eQTLs in lung tissue. These findings suggest that these SNPs may affect individual susceptibility to occupational exposures and ambient NO_2 exposure by altering gene expression levels. The other SNPs identified in the GWI study were not cis-eQTLs in lung tissue, but may affect individual susceptibility to occupational exposure and ambient NO_2 exposure via other mechanisms, i.e. changed protein structure, altered miRNA levels or methylation. Determining the functional mechanisms of the identified genetic variants will be an important focus in future studies.

Future perspectives

Epidemiological studies

In this thesis we have shown that environmental exposures including e-waste exposures, occupational exposures and ambient NO_2 exposures are associated with the level of lung function or the prevalence of respiratory symptoms. In addition, the GWI studies presented in this thesis have produced several novel loci which are associated with the development of respiratory symptoms. However, several important issues remain to be investigated. First, association between environmental exposures and the level of lung function and the prevalence of respiratory symptoms were studied in cross-sectional settings. In chapter 4 we investigated associations between e-waste exposures and the lower level of lung function in the cross-sectional study, which prevents us from drawing a causal relationship conclusion for the nature of cross-sectional study. Longitudinal studies are needed to assess associations between environmental exposures and the level of lung function and the development of respiratory symptoms in the future. Second, the genome-wide interaction studies presented in this thesis have focused on the prevalence of respiratory symptoms. Individuals with respiratory

symptoms are more apt to have lower level of lung function. In order to fully illustrate the pathways leading to the development of respiratory symptoms, future studies should focus on the level of lung function as well. Finally to answer the question why and how non-exposed individuals or low exposure individuals develop respiratory symptoms, associations between genes and their interactions on lung function levels and respiratory symptoms prevalence should be studied in a subgroup including only non-exposed individuals or low exposure individuals. GWA or GWI studies in these settings will teach us whether the same or different pathways underlie exposed and non-exposed respiratory symptoms such as occupational and non-occupational respiratory symptoms.

Post-GWAS functional studies

Genome-wide approaches provided the opportunity to identify genetic loci at which common variants influence symptom and disease risk or quantitative traits cross the entire genome. As an example, GWA and GWI studies could provide information on genes involved in previously unsuspected pathways, which could have been never considered in a candidate gene approach. Although GWA and GWI studies can provide a comprehensive list of significantly associated genetic variants, they are not designed to pinpoint the causative genes or gene mutations that underlie the complex trait or disease of interest, and only can explain a small fraction of the heritable component of symptoms/disease risk and the phenotypic variation that is observed. If they are additionally located outside the coding region of the gene, they also provide little evidence in terms of their functional significance (88). Therefore, several approaches have been used to explore and establish the functionality of identified SNPs and whether genes are causative (88, 89): (1) eQTL analysis is used to explore the association between gene variants and mRNA expression levels; (2) functional validation approach to establish the functional significance with in vitro and/or in vivo overexpression or

knock-down biological experiments; (3) bioinformatics combining epidemiologic, genomic, epigenetic, metablolomic and proteomic information for network analysis will help exploit integrative genomic approaches according to available databases. In spite of such attempts, identification of causative genes/mutations has been quite difficult and remains very challenging.

With the studies presented in this thesis we are the first assessing genome-wide interactions with environmental exposures such as occupational exposures and ambient NO_2 exposure in relation to the presence of respiratory symptoms. We identified several novel loci. Future challenges will be to understand the function of these loci. There are several options to go from identifying SNPs to assessing their functional mechanisms. The identified genes from our GWI studies still need investigation regarding their functionality in humans. In chapter 6 and chapter 7 of this thesis we have assessed whether identified SNPs were cis-eQTL in lung tissue, which gave us additional insight in potential pathways underlying the observed associations. Other approaches for post-GWA study analyses include re-sequencing, experimental models and epigenetic mechanisms (90, 91). Re-sequencing or fine mapping can be conducted sequence-based in follow-up of a GWA study, and can be used to identify the causal SNP that is in the linkage disequilibrium with the SNP associated with the outcome under study. In addition, fine mapping in different populations may also be used to identify rare variants associated with respiratory symptoms, thereby potentially explaining some of the missing heritability of the respiratory symptoms. Overexpression or knock-down of a gene in animals or cells may contribute to better understanding of biological pathways leading to a trait or a disease of interest. Epigenetic mechanisms including DNA methylation, histone modification and non-coding RNA (ncRNA) can contribute to gene regulation, and may mediate associations between SNPs and disease found in GWA or GWI studies.

Epigenetics

Epigenetics refers to heritable changes in gene expression that do not involve changes to the underlying DNA sequence, resulting in a change in phenotype without a change in genotype (92). Epigenetic mechanisms occur regularly and naturally, but can also be influenced by several factors like age, environment, and lifestyle and disease state (93). Epigenetic alterations mediate genomic adaption to the environment and can contribute to the development of disease phenotypes, as can genetic variants. GWA studies or GWI studies are not able to provide evidence for causative genetic effects of respiratory symptoms and diseases and can not fully explain the heritability of human. Genome-wide epigenetic studies can provide new insights into respiratory symptoms and diseases. Epigenetic mechanisms including DNA methylation, histone modification, nucleosome remodeling and non-coding RNA (Figure 1), are essential for normal development and differentiation, but also may be an important link between exposures and the development of respiratory symptoms or airway diseases (94-96). So far, most epigenetic studies focused on the effects of smoking on diseases, and genome-wide methylation patterns were shown to be associated with smoking status (97-99). In addition, cigarette smoke has been associated with both histone modifications (100) and altered expression of miRNA (101). Studying the role of epigenetic mechanisms in mediating associations between environmental exposures and gene expression and in the development of respiratory symptoms will be important in further studies. Such studies may improve our understanding of biological pathways underlying respiratory symptoms.

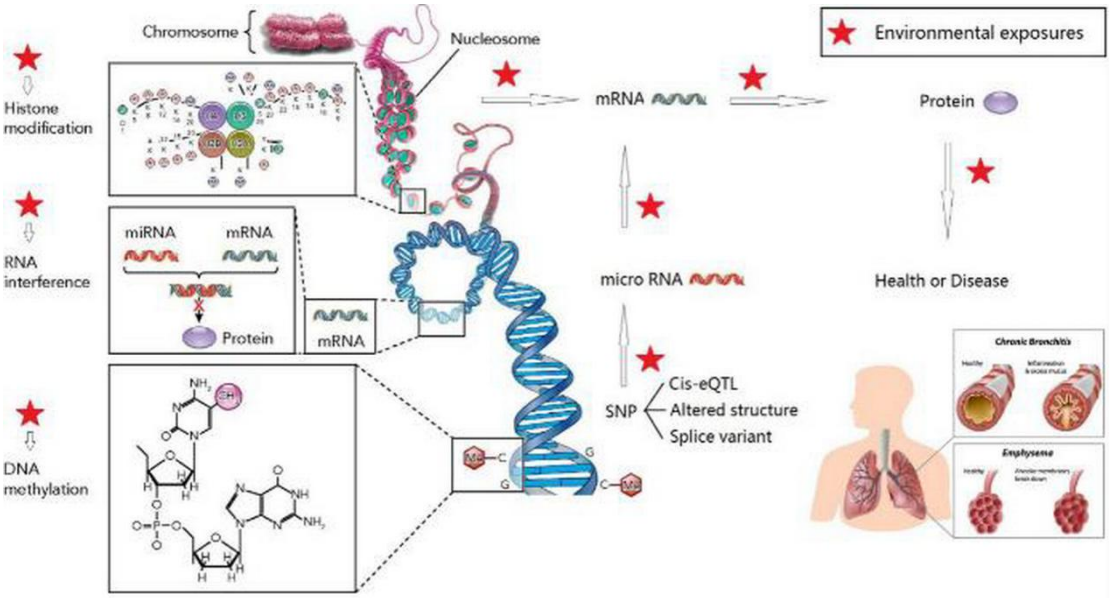

Figure 1. Major epigenetic mechanisms controlling gene expression (picture modified from Hagood (102)).

In summary, combining and analyzing genotype, methylation, gene expression and phenotypic data will be useful to uncover mechanisms underlying respiratory outcomes and diseases in future studies. This will require large computational power for genome-wide data. Soon, whole-genome methylation data of a subsample of 1,600 subjects from the LifeLines cohort study will be available, which will allow us to assess DNA methylation, and gene-environment interactions in relation to the development of respiratory symptoms or diseases. This may contribute to early identification of groups at risk for developing respiratory symptoms or disease, and might allow intervention at a very early stage of disease. The advances in GWI studies and in genome-wide epigenetic studies will probably contribute to the discovery of novel biological mechanisms underlying disease development and a better understanding of the etiology of respiratory diseases.

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CHAPTER 9

Samenvatting

Dankwoord

Currivulum Vitae

Samenvatting

Het doel van dit promotieonderzoek was om te onderzoeken of blootstelling aan verschillende stoffen uit de omgeving, zoals blootstelling aan elektronisch afval (e-waste), beroepsblootstellingen en luchtvervuiling, geassocieerd zijn met het niveau van longfunctie en met het voorkomen van klachten van de luchtwegen zoals hoesten en kortademigheid. Daarnaast waren we ook geïnteresseerd om de verschillen tussen individuen in genetische gevoeligheid voor de effecten van deze blootstellingen te evalueren. De studies beschreven in dit proefschrift tonen aan dat omgevingsblootstellingen geassocieerd zijn met een slechtere longfunctie en een hogere prevalentie van luchtwegklachten. We hebben verschillende genetische varianten geïdentificeerd die geassocieerd zijn met iemands persoonlijke gevoeligheid voor de effecten van beroepsblootstellingen en blootstelling aan stikstofdioxide (NO_2) in de buitenlucht.

In **hoofdstuk 1** wordt het onderzoek geïntroduceerd. De belangrijkste omgevingsrisicofactoren worden toegelicht en meer informatie wordt gegeven over de gebruikte uitkomstmaten. Daarnaast worden ook de methoden beschreven waarmee genetische associaties kunnen worden onderzocht. Ten slotte worden de gebruikte populaties in dit proefschrift geïntroduceerd.

Hoofdstuk 2 geeft een overzicht van andere onderzoeken die de nadelige gevolgen van e-waste op het menselijk lichaam hebben onderzocht. We hebben gekeken naar cross-sectionele associaties tussen blootstelling aan stoffen die in e-waste voorkomen, zoals giftige zware metalen en organische verontreinigende stoffen, en verminderde gezondheid, met inbegrip van endocriene toxiciteit, respiratoire toxiciteit, ontwikkelingstoxiciteit, reproductieve toxiciteit, neurotoxiciteit, en genetische toxiciteit. We hebben op basis van deze studie geconcludeerd dat zware metalen en organische verontreinigende stoffen afkomstig van e-waste zich ophopen in de omgeving, zoals in lucht, aarde, stof en water, en in het menselijk lichaam, zowel direct door inademing, inslikken en

absorptie door de huid, als indirect via de voedselketen. Blootstelling aan e-waste heeft nadelige gevolgen voor de gezondheid van de mens en kan leiden tot endocriene stoornissen, afnemen van de longfunctie, ontwikkelingsachterstand, beschadiging van het sperma, mentale achteruitgang, en DNA- en eiwitschade.

Hoofdstuk 3 geeft een overzicht van de nadelige effecten van een aantal zware metalen op de gezondheid van kinderen die wonen in gebieden waar e-waste wordt gerecycled, inclusief de gezondheid van de luchtwegen, hart, zenuwen, immuunsysteem, reproductiesysteem, skelet, en urinewegen. De zware metalen uit e-waste kunnen in de omgeving komen door verbranding, lozing en verwerking bij productiefaciliteiten. Kinderen zijn meer gevoelig voor deze zware metalen dan volwassenen om verschillende redenen, zoals een overschot aan blootstellingroutes, een hogere basaal metabolisme, en een lagere toxine eliminatiesnelheid. We vonden dat de concentratie van zware metalen in het milieu zoals lucht, stof, grond, sediment en water hoger is in gebieden waar e-waste gerecycled wordt dan in de referentiegebieden. Het wonen in e-waste recycle gebieden is geassocieerd met hogere concentraties van zware metalen in het bloed van de kinderen en blootstellingen aan zware metalen is geassocieerd met een verslechterde gezondheid.

Hoofdstuk 4 beschrijft een studie waarin we de relatie tussen wonen in een gebied waar e-waste gerecycled wordt en de concentratie van zware metalen in het bloed van heel jonge kinderen hebben onderzocht. Daarnaast hebben we de associaties onderzocht tussen de concentratie zware metalen in het bloed en het longfunctie niveau, en tussen wonen in e-waste recycle gebied en het longfunctie niveau in deze kinderen. We hebben geconcludeerd dat het wonen in een e-waste recycle gebied geassocieerd is met hogere bloed concentraties lood, bloedplaatjes en bloedplaatjesaggregatie, en lagere hemoglobine, hematocriet en longfunctie niveaus. Hemoglobine was daarnaast ook geassocieerd met hogere longfunctie niveaus.

Hoofdstuk 5 beschrijft een aantal genoom brede associatie studies (GWAS) waarin we beoogden om nieuwe genetische loci (SNPs) te identificeren, en te repliceren, die geassocieerd zijn met luchtweg gerelateerde symptomen zoals hoesten, kortademigheid en slijm opgeven. We hebben data van 7.976 personen van de eerste data uitgifte van de LifeLines cohort studie (LifeLines I) gebruikt voor de identificatie analyse, en hebben onze bevindingen proberen te repliceren in 5.260 personen van de tweede data uitgifte van de LifeLines cohort studie (LifeLines II) en 1.529 personen uit het Vlagtwedde-Vlaardingen cohort. We hebben één SNP geïdentificeerd, en gerepliceerd, die geassocieerd was met hoesten, namelijk rs16918212 gelegen in het gen *A2MP1*. Dit gen, een pseudogen, is niet eerder in de literatuur in verband gebracht met verminderde longfunctie of longziekten. Daarom is het niet erg waarschijnlijk dat dit gen betrokken is bij de ontwikkeling van luchtweg gerelateerde symptomen.

Hoofdstuk 6 beschrijft een genoom brede interactie studie (GWIS) waarin we beoogden om nieuwe genetische loci (SNPs) te identificeren die samenhangen met de gevoeligheid van een individu voor veelvoorkomende beroepsblootstellingen, zoals biologisch stof, mineraal stof, gassen, dampen, pesticiden, herbiciden, insecticiden, aromatische oplosmiddelen, gechloreerde oplosmiddelen, overige oplosmiddelen en zware metalen, op luchtweg gerelateerde symptomen, inclusief hoesten, kortademigheid en slijm. Als eerste hebben we een identificatie analyse uitgevoerd met de data van 7.976 personen van de eerste data uitgifte van de LifeLines cohort studie (LifeLines I). Hierna hebben we onze bevindingen proberen te repliceren door een meta-analyse uit te voeren op de resultaten van de analyses in 5.260 personen van de tweede data uitgifte van de LifeLines cohort studie (LifeLines II) en in 1.529 personen uit het Vlagtwedde-Vlaardingen cohort. Daarnaast hebben we onderzocht of deze SNPs geassocieerd waren met de gen-expressie in longweefsel (dit heet cis eQTLs (Expression quantitative trait loci)). We hebben 11 SNPs geïdentificeerd en gerepliceerd die in interactie waren met een van de beroepsblootstellingen. Verschillende geïdentificeerde SNPs liggen in genen die eerder geassocieerd zijn met de gezondheid van de longen,

onder andere *TMPRSS9, TOX3* en *ARHGAP18.* Drie van deze 11 SNPs waren cis eQTLs en geassocieerd met de gen expressie van de naburige genen *FCER1A, CHNA1* en *TIMM13*. Verder onderzoek zal moeten aantonen of de geïdentificeerde genen daadwerkelijk genetische loci zijn voor het ontwikkelen van luchtweg gerelateerde symptomen.

Hoofdstuk 7 beschrijft een genoom brede interactie studie (GWIS) waarin we beoogden om nieuwe genetische loci (SNPs) te identificeren die samenhangen met de gevoeligheid van een individu voor blootstelling aan NO_2 (een stof die voorkomt in de buitenlucht met name als gevolg van gemotoriseerd wegverkeer) en de luchtweg gerelateerde symptomen hoesten, kortademigheid, sputum opgeven en een piepende ademhaling. Interacties tussen SNPs en NO_2 blootstelling zijn onderzocht in 10,762 personen uit de LifeLines cohort studie. Daarnaast hebben we onderzocht of deze SNPs ook cis eQTLs waren in longweefsel. Verschillende geïdentificeerde loci liggen in genen die in de literatuur eerder geassocieerd zijn met longfunctie niveaus, zoals *ROCK2*, *GLS* en *KCTD10*. Vijftien van de 32 geïdentificeerde SNPs waren cis eQTLs in longweefsel en geassocieerd met gen expressie van nabijgelegen genen zoals *DBF4B, PRKAG2, EPS15, GRPEL1, MMAB, GLS, ZNF25, NCKAP5, GABRA2, ROCK2, MON1B* en *C14orf4*. De meest interessante SNP ligt in het gen *ROCK2*. We vonden dat personen die drager zijn van de minor allel van deze variant, een significant hogere *ROCK2* gen expressie in longweefsel hebben en ook een groter risico lopen om te piepen ten gevolge van blootstelling aan NO_2 vergeleken met personen die drager zijn van twee major allelen. Deze resultaten benadrukken de mogelijke rol van deze genen in de ontwikkeling van luchtweg gerelateerde symptomen. Deze resultaten moeten gerepliceerd worden in andere onafhankelijke cohorten om aan te kunnen tonen dat deze geïdentificeerde genen echte genetische loci zijn die de gevoeligheid voor luchtweg gerelateerde symptomen vergroten in combinatie met blootstelling aan NO_2, en of deze SNP-bij-NO_2 interacties consequent het risico op longziekten, zoals als astma en COPD, verhogen.

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Currivulum Vitae

About the author

Xiang Zeng was born on March 17, 1986 in Xishui County, Hubei Province, China. He got his bachelor degree (2008) in bioengineering from Yangtze University, and obtained his master degree (2011) in Medical Chemistry from Shantou University Medical College under the supervision of Prof. Daojun Liu, synthesizing amphiphilic cylindrical copolypeptide brushes as potential drug and gene carriers.

He applied the scholarship from University of Groningen and Shantou University Medical College for the PhD project presented in this thesis in the Department of Epidemiology, University Medical Center Groningen, University of Groningen. The project was focused on effects of environmental exposure on human health and interactions between genes and the environment in relation to respiratory symptoms. The process of project was under the supervision of Prof. H. Marike Boezen, Judith M. Vonk, and Porf. Xia Huo. The manuscript of this thesis was submitted to the reading committee in July 2016 and will be defended at October 12nd 2016.

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